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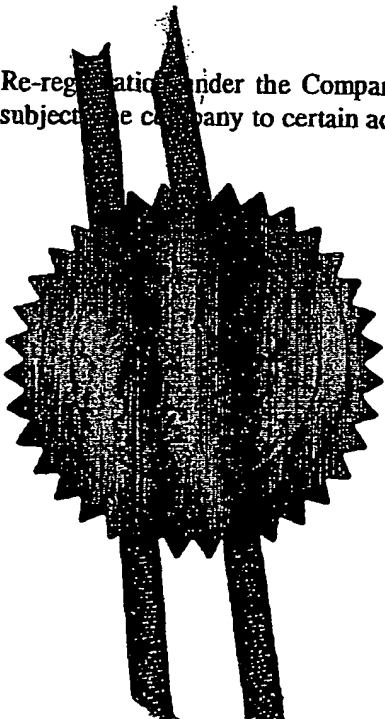
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Dated   19 January 2004

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NZAS-0212507

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Patents Form 1/77

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   Langebrogade 1
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   DK-1001 Copenhagen K
   Denmark

   Patents ADP number (if you know it)

   If the applicant is a corporate body, give the
   country/state of its incorporation

   Denmark

   5660873002

4. Title of the invention

   METHOD

5. Name of your agent (if you have one)

   D Young & Co

   "Address for service" in the United Kingdom
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   21 New Fetter Lane
   London
   EC4A 1DA

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Patents Form 1/77

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   Description   174

   Claim(s)   8

   Abstract   1

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    Signature(s)   *D Young & Co*   Date 24 December 2003

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METHOD

REFERENCE TO RELATED APPLICATIONS

Reference is made to the following related applications: United States Application

5   Serial Number 09/750,990 filed on 20 July 1999 and United States Application Serial Number 10/409,391. Each of these applications and each of the documents cited in each of these applications ("application cited documents"), and each document referenced or cited in the application cited documents, either in the text or during the prosecution of those applications, as well as all arguments in support of patentability

10   advanced during such prosecution, are hereby incorporated herein by reference. Various documents are also cited in this text ("herein cited documents"). Each of the herein cited documents, and each document cited or referenced in the herein cited documents, is hereby incorporated herein by reference.

15   FIELD OF INVENTION

The present invention relates to a method for the *in situ* production of an emulsifier within a foodstuff by use of a lipid acyltransferase.

20   The present invention further relates to a method for the *in situ* production of an emulsifier within a foodstuff by use of a lipid acyltransferase, wherein the method is such that the emulsifier is produced without increasing or without substantially increasing the free fatty acids in the foodstuff.

25   The present invention yet further relates to a method for the *in situ* production of at least two emulsifiers within a foodstuff by use of a lipid acyltransferase.

The present invention also relates to a method for the *in situ* production of a carbohydrate ester and/or a sterol ester and/or a stanol ester and/or a protein ester

30   and/or glycerol ester within a foodstuff by use of a lipid acyltransferase.

The present invention relates to a food enzyme composition and/or a feed enzyme composition, which contains a lipid acyltransferase, and the use of such a composition in the methods of the present invention.

5   The present invention further relates to an immobilised lipid acyltransferase as defined herein, which immobilised lipid acyltransferase may be used in bioconversion in a high water environment.

The present invention further relates to a method of identifying suitable lipid
10   acyltransferases in accordance with the present invention and to lipid acyltransferases so identified.

TECHNICAL BACKGROUND

15   The beneficial use of phospholipases and lipases (referred to as lipolytic enzymes, (EC. 3.1.1.x) used in food and/or feed industrial applications has been known for many years.

For instance, in EP 0 585 988 it is claimed that lipase addition to dough resulted in an
20   improvement in the antistaling effect. It is suggested that a lipase obtained from *Rhizopus arrhizus* when added to dough can improve the quality of the resultant bread when used in combination with shortening/fat. WO94/04035 teaches that an improved softness can be obtained by adding a lipase to dough without the addition of any additional fat/oil to the dough. Castello, P. ESEGP 89-10 Dec. 1999 Helsinki, shows
25   that exogenous lipases can modify bread volume.

Lipolytic enzymes hydrolyse one or more of the fatty acids from lipids present in the food which can result in the formation of powerful emulsifier molecules within the foodstuff which provide commercially valuable functionality. The molecules which
30   contribute the most significant emulsifier characteristics are the partial hydrolysis products, such as lyso-phospholipids, lyso-glycolipids, and mono-glyceride molecules. The polar lipid hydrolysis products, such as lyso-phospholipids and lyso-glycolipids

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are particularly advantageous. In bread making, such *in situ* derived emulsifiers can give equivalent functionality as emulsifiers, such as DATEM.

5   However, the activity of lipolytic enzymes also results in accumulation of free fatty acids, which can lead to detrimental functionality in the foodstuff. This inherent activity of lipolytic enzymes limits their functionality.

Numerous solutions to this problem have been attempted in the art. However, these result in a significant increase in free fatty acids in the foodstuff, particularly food

10   stuffs with high water content, including, but not limited to bread doughs and egg yolk.

Phospholipases, particularly phospholipase A2 (E.C. 3.1.1.4), have been used for many years for the treatment of egg or egg-based products (see US 4,034,124 and Dutihl & Groger 1981 J. Sci. Food Agric. 32, 451-458 for example). The phospholipase activity

15   during the treatment of egg or egg-based products results in the accumulation of polar lysolecithin, which can act as an emulsifier. Phospholipase treatment of egg or egg-based products can improve the stability, thermal stability under heat treatment such as pasteurisation and result in substantial thickening. Egg-based products may include, but are not limited to cake, mayonnaise, salad dressings, sauces, ice creams and the

20   like. Use of phospholipases results in the accumulation of free fatty acids. The accumulation of free fatty acids can result in significant off-flavour. In addition, the accumulation of free fatty acids can result in enhanced susceptibility to oxidation, and hence result in poor shelf-life, product discoloration and alteration of other critical food characteristics such as flavour and texture. Recently, lipolytic enzymes with

25   broader substrate specificity have been marketed for treatment of egg yolk and related food products. These have the advantage that, unlike most of the phospholipase A2s, they do not originate from a mammalian source. However, they result in significant accumulation of free fatty acids, not only due to the hydrolysis of phospholipids, but also triglycerides.

30

As mentioned above, another area where lipases have been extensively used is in the bakery industry. The use of phospholipases in baking dates bake to the early 1980s.

The substrate for lipases in wheat flour is 1.5-3% endogenous wheat lipids, which are a complex mixture of polar and non-polar lipids. The polar lipids can be divided into glycolipids and phospholipids. These lipids are built up of glycerol esterified with two

5   fatty acids and a polar group. The polar group contributes to surface activity of these lipids. Enzymatic cleavage of one of the fatty acids in these lipids leads to lipids with a much higher surface activity. It is well known that emulsifiers, such as DATEM, with high surface activity are very functional when added to dough.

10   However, the use of lipases (E.C. 3.1.1.X) in dough products may have a detrimental impact on yeast activity, and/or a negative effect on bread volume. The negative effect on bread volume is often explained by overdosing. Overdosing can lead to a decrease in gluten elasticity which results in a dough which is too stiff and thus results in reduced bread volumes. In addition, or alternatively, such lipases can degrade

15   shortening, oil or milk fat added to the dough, resulting in off-flavour in the dough and baked product. Overdosing and off flavour have been attributed to the accumulation of free fatty acids in the dough.

In EP 1 193 314, EP 0 977 869 and also in WO 01/39602, the use of lipolytic

20   enzymes active on glycolipids was reported to be particularly beneficial in application in bread making as the partial hydrolysis products the lyso-glycolipids were found to have very high emulsifier functionality, apparently resulting in a higher proportion of positive emulsifier functionality compared to the detrimental accumulation of free fatty acids. However, the enzymes were also found to have significant non selective

25   activity on triglyceride which resulted in unnecessarily high free fatty acid.

The same finding was reported in WO 00/32758, which disclosed lipolytic enzyme variants with enhanced activity on phospholipids and/or glycolipids, in addition to variants which had a preference for long rather than short chain fatty acids. This latter

30   feature, also disclosed in WO 01/39602, was deemed of particular importance to prevent the off-flavours associated with the accumulation of free short chain fatty acids. However, significant free fatty acids are produced.

The problem of high triglyceride activity was addressed in WO02/094123, where the use of lipolytic enzymes active on the polar lipids (i.e. glycolipids and phospholipids) in a dough, but substantially not active on triglycerides or 1-mono-glycerides is taught. However, significant free fatty acids are produced.

Some lipolytic enzymes have low or no activity on the lyso form of polar lipids (e.g. glycolipids/phospholipids). The use of such enzymes has been deemed preferable as they ensure the accumulation of the highly polar lyso-lipids, resulting in optimal functionality. Free fatty acids do however accumulate. This selective functionality is characteristic of phospholipase A2 enzymes, and the glycolipases disclosed in EP 0 977 869, EP 1 193 314, and WO01/39602. Variant enzymes of less selective lipolytic enzymes have been produced which have a lower activity on the lyso-polar lipids when compared to the parent enzyme (WO03/060112). However, significant free fatty acids are produced.

WO00/05396 teaches a process for preparing a foodstuff comprising an emulsifier, wherein food material is contacted with an enzyme such that an emulsifier is generated by the enzyme from a fatty acid ester and a second functional ingredient is generated from a second constituent. WO00/05396 teaches the use of in particular a lipase or esterase enzyme. Nowhere in WO00/05396 is the specific use of a lipid acyltransferase taught. In addition, in foodstuffs with high water content, the use of the esterases and lipases as taught in WO00/05396 would result in significant accumulation of free fatty acids.

A disadvantage associated with the use of lipases, including phospholipases and glycolipases, may be caused by the build-up of free fatty acids released from the lipids. Over the past couple of decades the use of lipolytic enzymes in foodstuffs has been limited due to the balance between the detrimental accumulation of free fatty acids and the production of the lyso-lipids which provide positive functionality. Although numerous strategies in the art have been attempted, some of which provided significant improvements in functionality, none have completely addressed and solved the

fundamental problem in the art, i.e. the significant accumulation of free fatty acids in foodstuffs prepared using lipolytic enzymes *in situ*.

The presence of high levels of free fatty acids (FFA) in raw materials or food products
5 is generally recognised as a quality defect and food processors and customers will usually include a maximum FFA level in the food specifications. The resulting effects of excess FFA levels can be in organoleptic and/or functional defects.

An obvious result of lipolysis is hydrolytic rancidity, with the formation of
10 characteristic "soapy" flavour. This "soapy" taste is especially acute with fatty acids of intermediate chain length (C8-C12) fatty acids which, although not present in high concentrations, may be important constituents of, for example, dairy products or vegetable oils. A more common organoleptic defect is due to the combined effects of lipolytic enzymes and oxidation processes. Unsaturated fatty acids are more
15 susceptible to enzymatic oxidation when unesterified than when esterified in acyl lipids.

Functional defects in food due to high FFA levels are recognised, but less readily explained. Without wishing to be bound by theory, the hydrolysis of unchanged lipids
20 to carboxylic acids will increase [H+] and produce carbonyl groups that can combine with other compounds or metal ions. Free fatty acids also combine proteins by hydrophobic interactions and can complex with starch during cooking. FFA may also interfere with the action of surface-active agents, such as polar lipids and emulsifiers. (Lipid in Cereal Technology, P.J. Barnes, Academic Press 1983.)
25

WO03/100044 discloses a class of acyl transferases known as PDATs (or ATWAX). These enzymes use a monoglyceride or a diglyceride as the acceptor molecule, and phosphatidyl cholin (PC) as the donor molecule to produce the following products: lyso phosphatidyl cholin and triacylglycerol and/or diacylglycerol.
30

In one embodiment, the present invention relates to improvements in the incorporation of whey proteins into food products, providing for improved yields without impairing the qualities – such as the texture – of the food compositions and products.

5 Cheese compositions are typically prepared from dairy liquids by processes that include treating the liquid with a coagulating or clotting agent. The coagulating agent may be a curding enzyme, an acid or a suitable bacterial culture, or it may include such a culture. The curd that results generally incorporates transformed casein, fats including natural butter fat, and flavourings that arise especially when a bacterial

10 culture is used. The curd may be separated from the liquid whey, which contains soluble proteins not affected by the coagulation and that therefore are not incorporated into the curd.

Whey is thus a by-product of manufacturing in commercial processes that produce

15 food products - such as cheeses. Traditionally, whey is disposed of as unused waste or used as fertiliser or animal feed or processed into a food ingredient.

The inability of whey proteins to be substantially retained in the curd is an important factor contributing to a lack of efficiency in the conventional production of dairy

20 products – such as cheese curds - and to a reduction in overall yield relating to the incorporation of all the protein solids that are present in the starting dairy liquids into resulting cheese curds.

There have been numerous attempts to include whey proteins in cheese e.g. by heat

25 treatment of the milk, heat treatment of whey, or by filtration – such as ultrafiltration.

There are also several descriptions of the use of specific proteases to induce aggregation of whey proteins. A serine protease derived from *Bacillus licheniformis* has been shown to have the ability to induce aggregation of whey proteins (US

30 5,523,237).

However, there remains many difficulties associated with adding whey proteins in processes such as the manufacture of cheeses. For example, incorporation of whey protein into cheeses is associated with a deterioration in the taste and mouth-feel of the product, and furthermore tends to interfere with curding and subsequent processing of the product. Proteases that have been previously reported that can be added to cheese milk for hydrolysis of whey proteins result in significant hydrolysis of the caseins as described by Madsen,J.S. & Qvist,K.B. (1997) Hydrolysis of milk protein by a *Bacillus licheniformis* protease specific for acidic amino acid residues. *J. Food Sci.* 62, 579-582.

Thus, there is a need in the art for methods and compositions that provide for the improved incorporation of whey protein into food products while maintaining organoleptic and other desirable properties. Such optimisation would result in increased efficiency, higher yields of food products, and reduced overall material costs.

Lipase:cholesterol acyltransferases have been known for some time (see for example Buckley – Biochemistry 1983, 22, 5490-5493). In particular, glycerophospholipid:cholesterol acyl transferases (GCATs) have been found, which like the plant and/or mammalian lecithin:cholesterol acyltransferases (LCATs), will catalyse fatty acid transfer between phosphatidylcholine and cholesterol.

Upton and Buckley (TIBS 20, May 1995 p 178-179) and Brumlik and Buckley (J. of Bacteriology Apr. 1996 p 2060-2064) teach a lipase/acyltransferase from *Aeromonas hydrophila* which has the ability to carry out acyl transfer to alcohol acceptors in aqueous media.

SUMMARY ASPECTS OF THE PRESENT INVENTION

According to a first aspect of the present invention there is provided a method of *in situ* production of an emulsifier in a foodstuff, wherein the method comprises the step of adding to the foodstuff a lipid acyltransferase as defined herein.

In a further aspect, the present invention provides a method of *in situ* production of an emulsifier in a foodstuff, wherein the method is such that the emulsifier is produced without increasing or without substantially increasing the free fatty acids in the foodstuff, and wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

In another aspect, the present invention provides a method of *in situ* production of an emulsifier and either a sterol ester and/or a stanol ester in a foodstuff, wherein the method is such that the emulsifier is produced without increasing or without substantially increasing the free fatty acids in the foodstuff, and wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

In another aspect, the present invention provides a method of *in situ* production of an emulsifier and either a sterol ester and/or a stanol ester in a foodstuff, wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

According to another aspect of the present invention there is provided a method for the *in situ* production of at least two emulsifiers in a foodstuff, wherein the method comprises the step of adding to the foodstuff a lipid acyltransferase.

According to a further aspect of the present invention there is provided a method of *in situ* production of at least two emulsifiers and either a sterol ester and/or a stanol ester in a foodstuff, wherein the method is such that the emulsifiers are produced without increasing or without substantially increasing the free fatty acids in the foodstuff, and wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

According to a further aspect of the present invention there is provided a method of *in situ* production of at least two emulsifiers and either a sterol ester and/or a stanol ester in a foodstuff, wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

In a further aspect, the present invention provides a method for the *in situ* production of a carbohydrate ester in a foodstuff, wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

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In another aspect, the present invention provides a method for the *in situ* production of a carbohydrate ester together with an emulsifier in a foodstuff, wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

10   In another aspect, the present invention provides a method of *in situ* production of an emulsifier, and one or more of a carbohydrate ester; a sterol ester; a stanol ester; a protein ester; a monoglyceride or a diglyceride in a foodstuff, and wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

15   According to a further aspect of the present invention there is provided a method of production of a foodstuff comprising an emulsifier, wherein the method comprises the step of adding to the foodstuff a lipid acyltransferase as defined herein.

In a further aspect, the present invention provides a method of production of a
20   foodstuff comprising an emulsifier, wherein the method is such that the emulsifier is produced without increasing or without substantially increasing the free fatty acids in the foodstuff, and wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

25   In another aspect, the present invention provides a method of the production of a foodstuff comprising an emulsifier and either a sterol ester and/or a stanol ester, wherein the method is such that the emulsifier is produced without increasing or without substantially increasing the free fatty acids in the foodstuff, and wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

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In another aspect, the present invention provides a method of the production of a foodstuff comprising an emulsifier and either a sterol ester and/or a stanol ester,

wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

According to a further aspect of the present invention there is provided a method for
5 the production of a foodstuff comprising at least two emulsifiers, wherein the method comprises the step of adding to the foodstuff a lipid acyltransferase.

According to a further aspect of the present invention there is provided a method of the production of a foodstuff comprising at least two emulsifiers and either a sterol ester
10 and/or a stanol ester, wherein the method is such that the emulsifiers are produced without increasing or without substantially increasing the free fatty acids in the foodstuff, and wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

15 According to a further aspect of the present invention there is provided a method of the production of a foodstuff comprising at least two emulsifiers and either a sterol ester and/or a stanol ester, wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

20 In a further aspect, the present invention provides a method for the production of a foodstuff comprising a carbohydrate ester, wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

In another aspect, the present invention provides a method for the production of a
25 foodstuff comprising a carbohydrate ester and an emulsifier, wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

In another aspect, the present invention provides a method of the production of a foodstuff comprising an emulsifier and one or more of a carbohydrate ester; a sterol
30 ester; a stanol ester; a protein ester; a monoglyceride or a diglyceride, and wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

In another aspect, the present invention provides use of a lipid acyltransferase to prepare from a food material a foodstuff comprising an emulsifier, wherein the emulsifier is generated from constituents of the food material by the lipid acyltransferase.

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In a further aspect, the present invention provides use of a lipid acyltransferase to prepare from a food material a foodstuff comprising an emulsifier, wherein the emulsifier is produced without increasing or without substantially increasing the free fatty acids in the foodstuff, and wherein the emulsifier is generated from constituents

10   of the food material by the lipid acyltransferase.

In another aspect, the present invention provides use of a lipid acyltransferase to prepare from a food material a foodstuff comprising an emulsifier and either a sterol ester and/or a stanol ester, wherein the emulsifier is produced without increasing or

15   without substantially increasing the free fatty acids in the foodstuff, and wherein the emulsifier and/or sterol ester and/or stanol ester is/are generated from constituents of the food material by the lipid acyltransferase.

In another aspect, the present invention provides use of a lipid acyltransferase to

20   prepare from a food material a foodstuff comprising an emulsifier and either a sterol ester and/or a stanol ester, wherein the emulsifier and/or sterol ester and/or stanol ester is/are generated from constituents of the food material by the lipid acyltransferase.

In another aspect, the present invention provides use of a lipid acyltransferase to

25   prepare from a food material a foodstuff comprising at least two emulsifiers, wherein the two emulsifiers are generated from constituents of the food material by the lipid acyltransferase.

According to a further aspect of the present invention there is provided use of a lipid

30   acyltransferase to prepare from a food material a foodstuff comprising at least two emulsifiers and either a sterol ester and/or a stanol ester, wherein the emulsifiers are produced without increasing or without substantially increasing the free fatty acids in

the foodstuff, and wherein one or both of the emulsifiers and/or the sterol ester and/or the stanol ester is/are generated from constituents of the food material by the lipid acyltransferase.

5   According to a further aspect of the present invention there is provided use of a lipid acyltransferase to prepare from a food material a foodstuff comprising at least two emulsifiers and either a sterol ester and/or a stanol ester, wherein one or both of the emulsifiers and/or the sterol ester and/or the stanol ester is/are generated from constituents of the food material by the lipid acyltransferase.

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In a further aspect, the present invention provides use of a lipid acyltransferase to prepare from a food material a foodstuff comprising a carbohydrate ester, wherein the carbohydrate ester is generated from constituents of the food material by the lipid acyltransferase.

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In another aspect, the present invention provides use of a lipid acyltransferase to prepare from a food material a foodstuff comprising at least a carbohydrate ester and a further emulsifier, wherein the carbohydrate ester and the emulsifier are generated from constituents of the food material by the lipid acyltransferase.

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In another aspect, the present invention provides use of a lipid acyltransferase to prepare from a food material a foodstuff comprising an emulsifier and one or more of a carbohydrate ester; a sterol ester; a stanol ester; a protein ester; a monoglyceride or a diglyceride, and wherein the emulsifier and/or the carbohydrate ester and/or the sterol

25   ester and/or the stanol ester and/or the protein ester and/or the monoglyceride and/or the diglyceride is/are generated from constituents of the food material by the lipid acyltransferase.

In accordance with a further aspect of the present invention there is provided a method

30   of the *in situ* production of an emulsifier, preferably a lysolecithin and a sterol ester in a egg based foodstuff, wherein the method is such that the emulsifier is produced without increasing or without substantially increasing the free fatty acids in the

foodstuff, and wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

In accordance with a further aspect of the present invention there is provided a method
5   of the *in situ* production of an emulsifier, preferably a lysolecithin, and a sterol ester in an egg based foodstuff, wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

In another aspect, the present invention provides a method of production of a egg
10 based foodstuff comprising an emulsifier, preferably a lysolecithin, and a sterol ester in an egg based foodstuff, wherein the emulsifier is produced without increasing or without substantially increasing the free fatty acids in the foodstuff, and wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

15 In another aspect, the present invention provides a method of production of a egg based foodstuff comprising an emulsifier, preferably a lysolecithin, and a sterol ester in an egg based foodstuff, wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

20 In a further aspect, the present invention further provides a foodstuff obtainable by, preferably obtained by, a method according to the present invention.

In another aspect the present invention further relates to a food enzyme composition and/or a feed enzyme composition, which contains a lipid acyltransferase, and the use
25 of such a composition in the methods of the present invention.

In further aspect, the present invention relates to an immobilised lipid acyltransferase as defined herein, which immobilised lipid acyltransferase may be used in bioconversion in a high water environment.
30
In accordance with a further aspect of the present invention there is provided a method of identifying a suitable lipid acyltransferase for use in accordance with the present

invention, comprising the steps of testing an enzyme of interest using one or more of the "Transferase Assay in a Low Water environment", the "Transferase Assay in High Water Egg Yolk" or the "Transferase Assay in Buffered Substrate", and selecting a lipid acyltransferase if it is one which has one or more of the following characteristics:

5   (a) when tested using the "Transferase Assay in a Low Water Environment", measured after a time period selected from 30, 20 or 120 minutes, has a relative transferase activity of at least 1%; (b) when tested using the "Transferase Assay in High Water Egg Yolk" in an egg yolk with 54% water, has up to 100% relative transferase activity; or (c) when tested using the "Transferase Assay in Buffered Substrate" has at

10   least 2% acyltransferase activity.

The present invention yet further provides a lipid acyltransferase identified using a method according to the present invention.

15   DETAILED ASPECTS OF THE PRESENT INVENTION

The term "lipid acyltransferase" as used herein means an enzyme which as well as having lipase activity (generally classified as E.C. 3.1.1.x in accordance with the Enzyme Nomenclature Recommendations (1992) of the Nomenclature Committee of

20   the International Union of Biochemistry and Molecular Biology) also has acyltransferase activity (generally classified as E.C. 2.3.1.x), whereby the enzyme is capable of transferring an acyl group from a lipid to one or more acceptor substrates, such as one or more of the following: a sterol; a stanol; a carbohydrate; a protein; glycerol.

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Preferably, the lipid acyltransferase for use in the methods and/or uses of the present invention is capable of transferring an acyl group from a lipid (as defined herein) to one or more of the following acyl acceptor substrates: a sterol, a stanol, a carbohydrate, a protein or subunits thereof, or a glycerol.

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For some aspects the the "acyl acceptor" according to the present invention may be any compound comprising a hydroxy group (-OH), such as for example, polyvalent

alcohols, including glycerol; sterol; stanols; carbohydrates; fruit acids and hydroxy acids including citric acid, tartaric acid, lactic acid and ascorbic acid; proteins or a sub-unit thereof, such as amino acids, protein hydrolysates and peptides (partly hydrolysed protein) for example; and mixtures and derivatives thereof. Preferably, the "acyl acceptor" according to the present invention is not water.

In one embodiment, the acyl acceptor is preferably not a monoglyceride and/or a diglyceride.

In one aspect, preferably the enzyme is capable of transferring an acyl group from a lipid to a sterol and/or a stanol.

In one aspect, preferably the enzyme is capable of transferring an acyl group from a lipid to a carbohydrate.

In one aspect, preferably the enzyme is capable of transferring an acyl group from a lipid to a protein.

In one aspect, preferably the enzyme is capable of transferring an acyl group from a lipid to glycerol.

In one aspect, the lipid acyltransferase may, as well as being able to transfer an acyl group from a lipid to a sterol and/or a stanol, additionally be able to transfer the acyl group from a lipid to one or more of the following: a carbohydrate, a protein, glycerol.

Preferably, the lipid substrate upon which the lipid acyltransferase according to the present invention acts is one or more of the following lipids: a phospholipid, such as a lecithin, e.g. phosphatidylcholine, a triacylglyceride, a diglyceride, or a glycolipid, such as digalactosyldiglyceride (DGDG) for example. This lipid substrate may be referred to herein as the "lipid acyl donor". The term lecithin as used herein encompasses phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and phosphatidylglycerol.

For some aspects, preferably the lipid substrate upon which the lipid acyltransferase acts is a phospholipid, such as lecithin, for example phosphatidylcholine.

For some aspects, preferably the lipid substrate is a glycolipid, such as DGDG for example.

Preferably the lipid substrate is a food lipid, that is to say a lipid component of a foodstuff.

For some aspects, preferably the lipid acyltransferase according to the present invention is incapable, or substantially incapable, of acting on a triglyceride and/or a 1-monoglyceride and/or 2-monoglyceride.

Suitably, the lipid substrate or lipid acyl donor may be one or more lipids present in one or more of the following substrates: fats, including lard, tallow and butter fat; oils including oils extracted from or derived from palm oil, sunflower oil, soya bean oil, safflower oil, cotton seed oil, ground nut oil, corn oil, olive oil, peanut oil, coconut oil, and rape seed oil. Lecithin from soya, rape seed or egg yolk is also a suitable lipid substrate. The lipid substrate may be an oat lipid or other plant based material containing galactolipids.

In one aspect the lipid acyl donor is preferably lecithin (such as phosphatidylcholine) in egg yolk.

For some aspects of the present invention, the lipid may be selected from lipids having a fatty acid chain length of from 8 to 22 carbons.

For some aspects of the present invention, the lipid may be selected from lipids having a fatty acid chain length of from 16 to 22 carbons, more preferably of from 16 to 20 carbons.

For some aspects of the present invention, the lipid may be selected from lipids having a fatty acid chain length of no greater than 14 carbons, suitably from lipids having a fatty acid chain length of from 4 to 14 carbons, suitably 4 to 10 carbons, suitably 4 to 8 carbons.

Suitably, the lipid acyltransferase according to the present invention may exhibit one or more of the following lipase activities: glycolipase activity (E.C. 3.1.1.26), triacylglycerol lipase activity (E.C. 3.1.1.3), phospholipase A2 activity (E.C. 3.1.1.4) or phospholipase A1 activity (E.C. 3.1.1.32). The term "glycolipase activity" as used herein encompasses "galactolipase activity".

Suitably, the lipid acyltransferase according to the present invention may have at least one or more of the following activities: glycolipase activity (E.C. 3.1.1.26) and/or phospholipase A1 activity (E.C. 3.1.1.32) and/or phospholipase A2 activity (E.C. 3.1.1.4).

For some aspects, the lipid acyltransferase according to the present invention may have at least glycolipase activity (E.C. 3.1.1.26).

Suitably, for some aspects the lipid acyltransferase according to the present invention may be capable of transferring an acyl group from a glycolipid and/or a phospholipid to one or more of the following acceptor substrates: a sterol, a stanol, a carbohydrate, a protein, glycerol.

For some aspects, preferably the lipid acyltransferase according to the present invention is capable of transferring an acyl group from a glycolipid and/or a phospholipid to a sterol and/or a stanol to form at least a sterol ester and/or a stanol ester.

For some aspects, preferably the lipid acyltransferase according to the present invention is capable of transferring an acyl group from a glycolipid and/or a phospholipid to a carbohydrate to form at least a carbohydrate ester.

For some aspects, preferably the lipid acyltransferase according to the present invention is capable of transferring an acyl group from a glycolipid and/or a phospholipid to a protein to form at least protein ester (or a protein fatty acid condensate).

For some aspects, preferably the lipid acyltransferase according to the present invention is capable of transferring an acyl group from a glycolipid and/or a phospholipid to glycerol to form at least a diglyceride and/or a monoglyceride.

For some aspects, preferably the lipid acyltransferase according to the present invention does not exhibit triacylglycerol lipase activity (E.C. 3.1.1.3).

In some aspects, the lipid acyltransferase may be capable of transferring an acyl group from a lipid to a sterol and/or a stanol. Thus, in one embodiment the "acyl acceptor" according to the present invention may be either a sterol or a stanol or a combination of both a sterol and a stanol.

Suitable sterol acyl acceptors include cholesterol and phytosterols, for example alpha-sitosterol, beta-sitosterol, stigmasterol, ergosterol, campesterol, 5,6-dihydrosterol, brassicasterol, alpha-spinasterol, beta-spinasterol, gamma-spinasterol, deltaspinasterol, fucosterol, dimosterol, ascosterol, serebisterol, episterol, anasterol, hyposterol, chondrillasterol, desmosterol, chalinosterol, poriferasterol, clionasterol, and other natural or synthetic isomeric forms and derivatives.

In one aspect of the present invention suitably more than one sterol and/or stanol may act as the acyl acceptor, suitably more than two sterols and/or stanols may act as the acyl acceptor. In other words, in one aspect of the present invention, suitably more than one sterol ester and/or stanol ester may be produced. Suitably, when cholesterol is the acyl acceptor one or more further sterols or one or more stanols may also act as the acyl acceptor. Thus, in one aspect, the present invention provides a method for the *in situ* production of both a cholesterol ester and at least one sterol or stanol ester in

combination. In other words, the lipid acyltransferase for some aspects of the present invention may transfer an acyl group from a lipid to both cholesterol and at least one further sterol and/or at least one stanol.

5   In one aspect, preferably the sterol acyl acceptor is one or more of the following: alpha-sitosterol, beta-sitosterol, stigmasterol, ergosterol and campesterol.

In one aspect, preferably the sterol acyl acceptor is cholesterol. When it is the case that cholesterol is the acyl acceptor for the lipid acyltransferase, the amount of free
10   cholesterol in the foodstuff is reduced as compared with the foodstuff prior to exposure to the lipid acyltransferase and/or as compared with an equivalent foodstuff which has not been treated with the lipid acyltransferase.

Suitable stanol acyl acceptors include phytostanols, for example beta-sitostanol or ss-
15   sitostanol.

In one aspect, preferably the sterol and/or stanol acyl acceptor is a sterol and/or a stanol other than cholesterol.

20   In some aspects, the foodstuff prepared in accordance with the present invention may be used to reduce blood serum cholesterol and/or to reduce low density lipoprotein. Blood serum cholesterol and low density lipoproteins have both been associated with certain diseases in humans, such as atherosclerosis and/or heart disease for example. Thus, it is envisaged that the foodstuffs prepared in accordance with the present
25   invention may be used to reduce the risk of such diseases.

Thus, in one aspect the present invention provides the use of a foodstuff according to the present invention for use in the treatment and/or prevention of atherosclerosis and/or heart disease.

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In a further aspect, the present invention provides a medicament comprising a foodstuff according to the present invention.

In a further aspect, the present invention provides a method of treating and/or preventing a disease in a human or animal patient which method comprising administering to the patient an effective amount of a foodstuff according to the present invention.

Suitably, the sterol and/or the stanol "acyl acceptor" may be found naturally within the foodstuff. Alternatively, the sterol and/or the stanol may be added to the foodstuff. When it is the case that a sterol and/or a stanol is added to the foodstuff, the sterol and/or stanol may be added before, simultaneously with, and/or after the addition of the lipid acyltransferase according to the present invention. Suitably, the present invention may encompass the addition of exogenous sterols/stanols, particularly phytosterols/phytostanols, to the foodstuff prior to or simultaneously with the addition of the enzyme according to the present invention.

For some aspects, one or more sterols present in the foodstuff may be converted to one or more stanols prior to or at the same time as the lipid acyltransferase is added according to the present invention. Any suitable method for converting sterols to stanols may be employed. For example, the conversion may be carried out by chemical hydrogenation for example. The conversion may be conducted prior to the addition of the lipid acyltransferase in accordance with the present invention or simultaneously with the addition of the lipid acyltransferase in accordance with the present invention. Suitably enzymes for the conversion of sterol to stanols are taught in WO00/061771.

Suitably the present invention may be employed to produce phytostanol esters *in situ* in a foodstuff. Phytostanol esters have increased solubility through lipid membranes, bioavailability and enhanced health benefits (see for example WO92/99640).

In some embodiments of the present invention the stanol ester and/or the sterol ester may be a flavouring and/or a texturiser. In which instances, the present invention encompasses the *in situ* production of flavourings and/or texturisers.

For some aspects of the present invention, the lipid acyltransferase according to the present invention may utilise a carbohydrate as the acyl acceptor. The carbohydrate acyl acceptor may be one or more of the following: a monosaccharide, a disaccharide, an oligosaccharide or a polysaccharide. Preferably, the carbohydrate is one or more of

5 the following: glucose, fructose, anhydrofructose, maltose, lactose, sucrose, galactose, xylose, xylooligosaccharides, arabinose, maltooligosaccharides and tagatose.

Suitably, the carbohydrate "acyl acceptor" may be found naturally within the foodstuff. Alternatively, the carbohydrate may be added to the foodstuff. When it is

10 the case that the carbohydrate is added to the foodstuff, the carbohydrate may be added before, simultaneously with, and/or after the addition of the lipid:sterol acyltransferase according to the present invention.

Carbohydrate esters can function as valuable emulsifiers in foodstuffs. Thus, when it

15 is the case that the enzyme functions to transfer the acyl group to a sugar, the invention encompasses the production of a second *in situ* emulsifier in the foodstuff.

In some embodiments, the lipid acyltransferase may utilise both a sterol and/or stanol and a carbohydrate as an acyl acceptor.

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The utilisation of lipid acyltransferase which can transfer the acyl group to a carbohydrate as well as to a sterol and/or a stanol is particularly advantageous for foodstuffs comprising eggs. In particular, the presence of sugars, in particular glucose, in eggs and egg products is often seen as disadvantageous. Egg yolk may comprise up

25 to 1% glucose. Typically, egg or egg based products may be treated with glucose oxidase to remove some or all of this glucose. However, in accordance with the present invention this unwanted sugar can be readily removed by "esterifying" the sugar to form a sugar ester.

30 For some aspects of the present invention, the lipid acyltransferase according to the present invention may utilise a protein as the acyl acceptor. Suitably, the protein may be one or more of the proteins found in a food product, for example in a dairy product

and/or a meat product. By way of example only, suitable proteins may be those found in curd or whey, such as lactoglobulin. Other suitable proteins include ovalbumin from egg, gliadin, glutenin, puroindoline, lipid transfer proteins from grains, and myosin from meat.

5

Thus in accordance with the present invention, one or more of the following advantageous properties can be achieved: *in situ* production of an emulsifier without an increase in free fatty acids; a reduction in the accumulation of free fatty acids in the foodstuff; a reduction in free cholesterol levels in the foodstuff; an increase in sterol

10 esters and/or stanol esters; a reduction in blood serum cholesterol and/or low density lipoproteins; an increase in carbohydrate esters; a reduction in unwanted free carbohydrates.

An advantage of the present invention is that the emulsifier(s) is/are prepared *in situ* in

15 the foodstuff without an increase, or a substantial, increase, in the free fatty acid content of the foodstuff. The production of free fatty acids can be detrimental to foodstuffs. In particular, free fatty acids have been linked with off-odours and/or off-flavours in foodstuffs, as well other detrimental effects, including a soapy taste in cheese for instance. Preferably, the method according to the present invention results

20 in the *in situ* preparation of an emulsifier(s) wherein the accumulation of free fatty acids is reduced and/or eliminated. Without wishing to be bound by theory, in accordance with the present invention the fatty acid which is removed from the lipid is transferred by the lipid acyltransferase to a sterol and/or a stanol. Thus, the overall level of free fatty acids in the foodstuff does not increase or increases only to an

25 insignificant degree. This is in sharp contradistinction to the situation when lipases (E.C. 3.1.1.x) are used to produce emulsifiers *in situ*. In particular, the use of lipases can result in an increased amount of free fatty acid in the foodstuff, which can be detrimental. In accordance with the present invention, the accumulation of free fatty acids is reduced and/or eliminated when compared with the amount of free fatty acids

30 which would have been accumulated had a lipase enzyme, in particular a phospholipase A_2 enzyme, been used in place of the lipid acyltransferase in accordance with the present invention.

The utilisation of a lipid acyltransferase which can transfer the acyl group to a sterol and/or stanol may be particularly advantageous for foodstuffs comprising eggs. In particular, it has been found that an egg-based product with significantly better properties can be obtained following treatment with a lipid acyltransferase as defined herein compared with egg-based products treated with conventional phospholipases, such as LipopanF® (Novozymes A/S, Denmark)), Lecitase Ultra® (Novozymes A/S, Denmark) or Lipomod 22 L from Biocatalysts, for instance.

Preferably, the lipid acyltransferase enzyme according to the present invention may be characterised using the following criteria:

(i)   the enzyme possesses acyl transferase activity which may be defined as ester transfer activity whereby the acyl part of an original ester bond of a lipid acyl donor is transferred to a sterol and/or a stanol acyl acceptor to form a new ester, i.e. a sterol ester and/or a stanol ester; and

(ii)   the enzyme comprises the amino acid sequence motif GDSX, wherein X is one or more of the following amino acid residues L, A, V, I, F, Y, H, Q, T, N, M or S.

Preferably, X of the GDSX motif is L. Thus, preferably the enzyme according to the present invention comprises the amino acid sequence motif GSDL.

The GDSX motif is comprised of four conserved amino acids. Preferably, the serine within the motif is a catalytic serine of the lipid acyltransferase enzyme. Suitably, the serine of the GDSX motif may be in a position corresponding to Ser-16 in *Aeromonas hydrophila* lipolytic enzyme taught in Brumlik & Buckley (Journal of Bacteriology Apr. 1996, Vol. 178, No. 7, p 2060-2064).

To determine if a protein has the GDSX motif according to the present invention, the sequence is preferably compared with the hidden markov model profiles (HMM profiles) of the pfam database.

Pfam is a database of protein domain families. Pfam contains curated multiple sequence alignments for each family as well as profile hidden Markov models (profile HMMs) for identifying these domains in new sequences. An introduction to Pfam can be found in Bateman A *et al* (2002) Nucleic Acids Res. 30; 276-280. Hidden Markov

5   models are used in a number of databases that aim at classifying proteins, for review see Bateman A and Haft DH (2002) Brief Bioinform 3; 236-245.

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&list_uids
=12230032&dopt=Abstract

10   http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&list_uids
=11752314&dopt=Abstract

For a detailed explanation of hidden Markov models and how they are applied in the Pfam database see Durbin R, Eddy S, and Krogh A (1998) Biological sequence

15   analysis; probabilistic models of proteins and nucleic acids. Cambridge University Press, ISBN 0-521-62041-4. The Hammer software package can be obtained from Washington University, St Louis, USA.

Alternatively, the GDSX motif can be identified using the Hammer software package,

20   the instructions are provided in Durbin R, Eddy S, and Krogh A (1998) Biological sequence analysis; probabilistic models of proteins and nucleic acids. Cambridge University Press, ISBN 0-521-62041-4 and the references therein, and the HMMER2 profile provided within this specification.

25   The PFAM database can be accessed, for example, through several servers which are currently located at the following websites.
http://www.sanger.ac.uk/Software/Pfam/index.shtml
http://pfam.wustl.edu/
http://pfam.jouy.inra.fr/

30   http://pfam.cgb.ki.se/

The database offers a search facility where one can enter a protein sequence. Using the default parameters of the database the protein sequence will then be analysed for the presence of Pfam domains. The GDSX domain is an established domain in the database and as such its presence in any query sequence will be recognised . The database will return the alignment of the Pfam00657 consensus sequence to the query sequence.

A multiple alignment, including *Aeromonas salmonicida* or *Aeromonas hydrophila* can be obtained by:

a) manual

obtain an alignment of the protein of interest with the Pfam00657 consensus sequence and obtain an alignment of P10480 with the Pfam00657 consensus sequence following the procedure described above;

Or

b) through the database

After identification of the Pfam00657 consensus sequence the database offers the option to show an alignment of the query sequence to the seed alignment of the Pfam00657 consensus sequence. P10480 is part of this seed alignment and is indicated by GCAT_AERHY. Both the query sequence and P10480 will be displayed in the same window.

The *Aeromonas hydrophila* reference sequence:

The residues of *Aeromonas hydrophila* GDSX lipase are numbered in the NCBI file P10480, the numbers in this text refer to the numbers given in that file which in the present invention is used to determine specific amino acids residues which, in a preferred embodiment are present in the lipid acyltransferase enzymes of the invention.

The Pfam alignment was performed (Figure 33 and 34):

The following conserved residues can be recognised and in a preferable embodiment may be present in the enzymes for use in the compositions and methods of the invention;

5   Block 1 - GDSX block
     hid hid hid hid Gly Asp Ser hid
      28 29 30 31 32 33 34 35

     Block 2 - GANDY block
10   hid Gly hid Asn Asp hid
     130 131 132 133 134 135

     Block 3 - HPT block
     His
15   309

The Where 'hid' means a hydrophobic residue selected from Met, Ile, Leu, Val, Ala, Gly, Cys, His, Lys, Trp, Tyr, Phe.

20

Preferably the lipid acyltransferase enzyme for use in the compositions/methods of the invention can be aligned using the Pfam00657 consensus sequence.

Preferably, a positive match with the hidden markov model profile (HMM profile) of
25   the pfam00657 domain family indicates the presence of the GDSL or GDSX domain according to the present invention.

Preferably when aligned with the Pfam00657 consensus sequence the lipid acyltransferase for use in the compositions/methods of the invention have at least one,
30   preferably more than one, preferably more than two, of the following, a GDSx block, a GANDY block, a HPT block. Suitably, the lipid acyltransferase may have a GDSx block and a GANDY block. Alternatively, the enzyme may have a GDSx block and a HPT block. Preferably the enzyme comprises at least a GDSx block.

Preferably, when aligned with the Pfam00657 consensus sequence the enzyme for use in the compositions/methods of the invention have at least one, preferably more than one, preferably more than two, preferably more than three, preferably more than four, preferably more than five, preferably more than six, preferably more than seven, preferably more than eight, preferably more than nine, preferably more than ten, preferably more than eleven, preferably more than twelve, preferably more than thirteen, preferably more than fourteen, of the following amino acid residues when compared to the reference *A.hydrophilia* polypeptide sequence, namely SEQ ID No. 32: 28hid, 29hid, 30hid, 31hid, 32gly, 33Asp, 34Ser, 35hid, 130hid, 131Gly, 132Hid, 133Asn, 134Asp, 135hid, 309His

The pfam00657 GDSX domain is a unique identifier which distinguishes proteins possessing this domain from other enzymes.

The pfam00657 consensus sequence is presented in Figure 1 as SEQ ID No. 1. This is derived from the identification of the pfam family 00657, database version 6, which may also be referred to as pfam00657.6 herein.

The consensus sequence may be updated by using further releases of the pfam database.

For example, Figures 33 and 34 show the pfam alignment of family 00657, from database version 11, which may also be referred to as pfam00657.11 herein.

The presence of the GDSx, GANDY and HPT blocks are found in the pfam family 00657 from both releases of the database. Future releases of the pfam database can be used to identify the pfam family 00657.

Preferably, the lipid acyltransferase enzyme according to the present invention may be characterised using the following criteria:

(i)   the enzyme possesses acyl transferase activity which may be defined as ester transfer activity whereby the acyl part of an original ester bond of

a lipid acyl donor is transferred to a sterol and/or a stanol acyl acceptor to form a new ester, i.e. a sterol ester and/or a stanol ester;

(ii)   the enzyme comprises the amino acid sequence motif GDSX, wherein X is one or more of the following amino acid residues L, A, V, I, F, Y, H, Q, T, N, M or S.;

(iii)   the enzyme comprises His-309 or comprises a histidine residue at a position corresponding to His-309 in the *Aeromonas hydrophila* lipolytic enzyme shown in Figure 2 (SEQ ID No. 2 or SEQ ID No. 32).

Preferably, the amino acid residue of the GDSX motif is L.

In SEQ ID No. 2 or SEQ ID No. 32 the first 18 amino acid residues form a signal sequence. His-309 of the full length sequence, that is the protein including the signal sequence, equates to His-291 of the mature part of the protein, i.e. the sequence without the signal sequence.

Preferably, the lipid acyltransferase enzyme according to the present invention comprises the following catalytic triad: Ser-34, Asp-134 and His-309 or comprises a serine residue, an aspartic acid residue and a histidine residue, respectively, at positions corresponding to Ser-34, Asp-134 and His-309 in the *Aeromonas hydrophila* lipolytic enzyme shown in Figure 2 (SEQ ID No. 2) or Figure 28 (SEQ ID No. 32). As stated above, in the sequence shown in SEQ ID No. 2 or SEQ ID No. 32 the first 18 amino acid residues form a signal sequence. Ser-34, Asp-134 and His-309 of the full length sequence, that is the protein including the signal sequence, equate to Ser-16, Asp-116 and His-291 of the mature part of the protein, i.e. the sequence without the signal sequence. In the pfam00657 consensus sequence, as given in Figure 1 (SEQ ID No. 1) the active site residues correspond to Ser-7, Asp-157 and His-348.

Preferably, the lipid acyltransferase enzyme according to the present invention may be characterised using the following criteria:

(i)   the enzyme possesses acyl transferase activity which may be defined as ester transfer activity whereby the acyl part of an original ester bond of

a first lipid acyl donor is transferred to a sterol and/or a stanol acyl acceptor to form a new ester, i.e. a sterol ester and/or a stanol ester; and

(ii)   the enzyme comprises at least Gly-32, Asp-33, Ser-34, Asp-134 and His-309 or comprises glycine, aspartic acid, serine, aspartic acid and histidine residues at positions corresponding to Gly-32, Asp-33, Ser-34, Asp-134 and His-309, respectively, in the *Aeromonas hydrophila* lipolytic enzyme shown in Figure 2 (SEQ ID No. 2) or Figure 28 (SEQ ID No. 32).

Suitably, the lipid acyltransferase enzyme according to the present invention may be obtainable, preferably obtained, from organisms from one or more of the following genera: *Aeromonas, Streptomyces, Saccharomyces, Lactococcus, Mycobacterium, Streptococcus, Lactobacillus, Desulfitobacterium, Bacillus, Campylobacter, Vibrionaceae, Xylella, Sulfolobus, Aspergillus, Schizosaccharomyces, Listeria, Neisseria, Mesorhizobium, Ralstonia, Xanthomonas* and *Candida*.

Suitably, the lipid acyltransferase enzyme according to the present invention may be obtainable, preferably obtained, from one or more of the following organisms: *Aeromonas hydrophila, Aeromonas salmonicida, Streptomyces coelicolor, Streptomyces rimosus, Mycobacterium, Streptococcus pyogenes, Lactococcus lactis, Streptococcus pyogenes, Streptococcus thermophilus, Lactobacillus helveticus, Desulfitobacterium dehalogenans, Bacillus sp, Campylobacter jejuni, Vibrionaceae, Xylella fastidiosa, Sulfolobus solfataricus, Saccharomyces cerevisiae, Aspergillus terreus, Schizosaccharomyces pombe, Listeria innocua, Listeria monocytogenes, Neisseria meningitidis, Mesorhizobium loti, Ralstonia solanacearum, Xanthomonas campestris, Xanthomonas axonopodis* and *Candida parapsilosis*.

In one aspect, preferably the lipid acyltransferase enzyme according to the present invention is obtainable, preferably obtained, from one or more of *Aeromonas hydrophila* or *Aeromonas salmonicida*.

Suitably, the lipid acyltransferase enzyme according to the present invention comprises one or more of the following amino acid sequences:

(i)   the amino acid sequence shown as SEQ ID No. 2 (see Figure 2)

(ii)   the amino acid sequence shown as SEQ ID No. 3 (see Figure 3)

5   (iii)   the amino acid sequence shown as SEQ ID No. 4 (see Figure 4)

(iv)   the amino acid sequence shown as SEQ ID No. 5 (see Figure 5)

(v)   the amino acid sequence shown as SEQ ID No. 6 (see Figure 6)

(vi)   the amino acid sequence shown as SEQ ID No. 12 (see Figure 14)

(vii)   the amino acid sequence shown as SEQ ID No. 20 (Figure 16)

10   (viii)   the amino acid sequence shown as SEQ ID No. 22 (Figure 18)

(ix)   the amino acid sequence shown as SEQ ID No. 24 (Figure 20)

(x)   the amino acid sequence shown as SEQ ID No. 26 (Figure 22)

(xi)   the amino acid sequence shown as SEQ ID No. 28 (Figure 24)

(xii)   the amino acid sequence shown as SEQ ID No. 30 (Figure 26)

15   (xiii)   the amino acid sequence shown as SEQ ID No. 32 (Figure 28)

(xiv) the amino acid sequence shown as SEQ ID No. 34 (Figure 30) or

an amino acid sequence which has 75% or more identity with any one of the sequences shown as SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 12, SEQ ID No. 20, SEQ ID No. 22, SEQ ID No. 24, SEQ ID No. 26,

20   SEQ ID No. 28, SEQ ID No. 30, SEQ ID No. 32, or SEQ ID No. 34.

Suitably, the lipid acyltransferase enzyme according to the present invention comprises either the amino acid sequence shown as SEQ ID No. 2 or as SEQ ID No. 3 or SEQ ID No. 32 or SEQ ID No. 34 or comprises an amino acid sequence which has 75% or

25   more, preferably 80% or more, preferably 85% or more, preferably 90% or more, preferably 95% or more, identity with the amino acid sequence shown as SEQ ID No. 2 or the amino acid sequence shown as SEQ ID No. 3 or the amino acid sequence shown as SEQ ID No. 32 or the amino acid sequence shown as SEQ ID No. 34.

30   For the purposes of the present invention, the degree of identity is based on the number of sequence elements which are the same. The degree of identity in accordance with the present invention may be suitably determined by means of computer programs

known in the art, such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, US53711) (Needleman & Wunsch (1970), J. of Molecular Biology 48, 443-45) using the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1.

Suitably the lipid acyltransferase enzyme according to the present invention comprises an amino acid sequence which has 80% or more, preferably 85% or more, more preferably 90% or more and even more preferably 95% or more identity with any one of the sequences shown as SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 12, SEQ ID No. 20, SEQ ID No. 22, SEQ ID No. 24, SEQ ID No. 26, SEQ ID No. 28, SEQ ID No. 30, SEQ ID No. 32, or SEQ ID No. 34.

Suitably, the lipid acyltransferase enzyme according to the present invention comprises one or more of the following amino acid sequences:

(a) an amino acid sequence shown as amino acid residues 1-100 of SEQ ID No. 2 or SEQ ID No. 32;

(b) an amino acid sequence shown as amino acids residues 101-200 of SEQ ID No. 2 or SEQ ID No. 32;

(c) an amino acid sequence shown as amino acid residues 201-300 of SEQ ID No. 2 or SEQ ID No. 32; or

(d) an amino acid sequence which has 75% or more, preferably 85% or more, more preferably 90% or more, even more preferably 95% or more identity to any one of the amino acid sequences defined in (a)-(c) above.

Suitably, the lipid acyltransferase enzyme according to the present invention comprises one or more of the following amino acid sequences:

(a) an amino acid sequence shown as amino acid residues 28-39 of SEQ ID No. 2 or SEQ ID No. 32;

(b) an amino acid sequence shown as amino acids residues 77-88 of SEQ ID No. 2 or SEQ ID No. 32;

(c) an amino acid sequence shown as amino acid residues 126-136 of SEQ ID No. 2 or SEQ ID No. 32;

(d) an amino acid sequence shown as amino acid residues 163-175 of SEQ ID No. 2 or SEQ ID No. 32;

5   (e) an amino acid sequence shown as amino acid residues 304-311 of SEQ ID No. 2 or SEQ ID No. 32; or

(f) an amino acid sequence which has 75% or more, preferably 85% or more, more preferably 90% or more, even more preferably 95% or more identity to any one of the amino acid sequences defined in (a)-(e) above.

10

Suitably, the lipid acyltransferase enzyme according to the present invention may comprise an amino acid sequence produced by the expression of one or more of the following nucleotide sequences:

(a) the nucleotide sequence shown as SEQ ID No. 7 (see Figure 9);

15   (b) the nucleotide sequence shown as SEQ ID No. 8 (see Figure 10);

- (c) the nucleotide sequence shown as SEQ ID No. 9 (see Figure 11);

(d) the nucleotide sequence shown as SEQ ID No. 10 (see Figure 12);

(e) the nucleotide sequence shown as SEQ ID No. 11 (see Figure 13);

(f) the nucleotide sequence shown as SEQ ID No. 13 (see Figure 15);

20   (g) the nucleotide sequence shown as SEQ ID No. 21 (see Figure 17);

(h) the nucleotide sequence shown as SEQ ID No. 23 (see Figure 19);

(i) the nucleotide sequence shown as SEQ ID No. 25 (see Figure 21);

(j) the nucleotide sequence shown as SEQ ID No. 27 (see Figure 23);

(k) the nucleotide sequence shown as SEQ ID No. 29 (see Figure 25);

25   (l) the nucleotide sequence shown as SEQ ID No. 31 (see Figure 27);

(m) the nucleotide sequence shown as SEQ ID No. 33 (see Figure 29);

(n) the nucleotide sequence shown as SEQ ID No. 35 (see Figure 31);

(o) or

a nucleotide sequence which has 75% or more identity with any one of the sequences

30   shown as SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 13, SEQ ID No. 21, SEQ ID No. 23, SEQ ID No. 25, SEQ ID No. 27, SEQ ID No. 29, SEQ ID No. 31, SEQ ID No. 33 or SEQ ID No. 35.

Suitably the nucleotide sequence may have 80% or more, preferably 85% or more, more preferably 90% or more and even more preferably 95% or more identity with any one of the sequences shown as SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID
5   No. 10, SEQ ID No. 11, SEQ ID No. 13, SEQ ID No. 21, SEQ ID No. 23, SEQ ID No. 25, SEQ ID No. 27, SEQ ID No. 29, SEQ ID No. 31, SEQ ID No. 33 or SEQ ID No. 35.

In one aspect, the lipid acyltransferase according to the present invention may be a
10 lecithin:cholesterol acyltransferases (LCAT) or variant thereof (for example a variant made by molecular evolution)

Suitable LCATs are known in the art and may be obtainable from one or more of the following organisms for example: mammals, rat, mice, chickens, *Drosophila*
15 *melanogaster*, plants, including *Arabidopsis* and *Oryza sativa*, nematodes, fungi and yeast.

In one embodiment the lipid acyltransferase enzyme according to the present invention may be the lipid acyltransferase obtainable, preferably obtained, from the *E. coli*
20 strains TOP 10 harbouring pPet12aAhydro and pPet12aASalmo deposited by Danisco A/S of Langebrogade 1, DK-1001 Copenhagen K, Denmark under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the purposes of Patent Procedure at the National Collection of Industrial, Marine and Food Bacteria (NCIMB) 23 St. Machar Street, Aberdeen Scotland, GB on 22 December 2003 under
25 accession numbers NICMB 41204 and NCIMB 41205, respectively.

Preferably, when carrying out a method according to the present invention the product is produced without increasing or substantially increasing the free fatty acids in the foodstuff.
30

The term "transferase" as used herein is interchangeable with the term "lipid acyltransferase".

Suitably, the lipid acyltransferase as defined herein catalyses one or more of the following reactions: interesterification, transesterification, alcoholysis, hydrolysis.

5   The term "bioconversion" refers to the modification and/or synthesis of organic compounds by enzyme catalysis, to product organic compounds. Bioconversion may take place *in vitro* or *in vivo*.

The term "interesterification" refers to the enzymatic catalysed transfer of acyl groups

10   between a lipid donor and lipid acceptor, wherein the lipid donor is not a free acyl group.

The term "transesterification" as used herein means the enzymatic catalysed transfer of an acyl group from a lipid donor (other than a free fatty acid) to an acyl acceptor (other

15   than water).

As used herein, the term "alcoholysis" refers to the enzymatic cleavage of a covalent bond of an acid derivative by reaction with an alcohol ROH so that one of the products combines with the H of the alcohol and the other product combines with the OR group

20   of the alcohol.

As used herein, the term "alcohol" refers to an alkyl compound containing a hydroxyl group.

25   As used herein, the term "hydrolysis" refers to the enzymatic catalysed transfer of an acyl group from a lipid to the OH group of a water molecule. Acyl transfer which results from hydrolysis requires the separation of the water molecule.

The term "without increasing or without substantially increasing the free fatty acids"

30   as used herein means that preferably the lipid acyl transferase according to the present invention has 100% transferase activity (i.e. transfers 100% of the acyl groups from an acyl donor onto the acyl acceptor, with no hydrolytic activity); however, the enzyme

may transfer less than 100% of the acyl groups present in the lipid acyl donor to the acyl acceptor. In which case, preferably the acyltransferase activity accounts for at least 5%, more preferably at least 10%, more preferably at least 20%, more preferably at least 30%, more preferably at least 40%, more preferably 50%, more preferably at

5   least 60%, more preferably at least 70%, more preferably at least 80%, more preferably at least 90% and more preferably at least 98% of the total enzyme activity. The % transferase activity (i.e. the transferase activity as a percentage of the total enzymatic activity) may be determined by the following protocol:

10   *Protocol for the determination of % acyltransferase activity:*

A foodstuff to which a lipid acyltransferase according to the present invention has been added may be extracted following the enzymatic reaction with $CHCl_3:CH_3OH$ 2:1 and the organic phase containing the lipid material is isolated and analysed by

15   GLC and HPLC according to the procedure detailed hereinbelow. From the GLC and HPLC analyses the amount of free fatty acids and one or more of sterol/stanol esters; carbohydrate esters, protein esters; diglycerides; or monoglycerides are determined. A control foodstuff to which no enzyme according to the present invention has been added, is analysed in the same way.

20   *Calculation:*

From the results of the GLC and HPLC analyses the increase in free fatty acids and sterol/stanol esters and/or carbohydrate esters and/or protein esters and/or diglycerides and/or monoglycerides can be calculated:

Δ % fatty acid = % Fatty acid(enzyme) - % fatty acid(control); Mv fatty acid   =

25   average molecular weight of the fatty acids;

A = Δ % sterol ester/Mv sterol ester (where Δ % sterol ester   = % sterol/stanol ester(enzyme) - % sterol/stanol ester(control) and Mv sterol ester = average molecular weight of the sterol/stanol esters) – applicable where the acyl acceptor is a sterol and/or stanol;

30   B = Δ % carbohydrate ester/Mv carbohydrate ester (where Δ % carbohydrate ester = % carbohydrate ester(enzyme) - % carbohydrate ester(control) and Mv carbohydrate ester

= average molecular weight of the carbohydrate ester) - applicable where the acyl acceptor is a carbohydrate;

C = Δ % protein ester/Mv protein ester (where Δ % protein ester = % protein ester(enzyme) - % protein ester(control) and Mv protein ester = average molecular weight of the protein ester) – applicable where the acyl acceptor is a protein; and

D = absolute value of diglyceride and/or monoglyceride/Mv di/monoglyceride (where Δ% diglyceride and/or monoglyceride = % diglyceride and/or monoglyceride (enzyme) - % diglyceride and/or monoglyceride (control) and Mv di/monoglyceride = average molecular weight of the diglyceride and/or monoglyceride) – applicable where the acyl acceptor is glycerol.

The transferase activity is calculated as a percentage of the total enzymatic activity:

$$\% \text{ transferase activity} = \frac{A^* + B^* + C^* + D^*}{A^* + B^* + C^* + D^* + \Delta\% \text{ fatty acid/(Mv fatty acid)}} \times 100$$

* - delete as appropriate.

If the free fatty acids are increased in the foodstuff they are preferably not increased substantially, i.e. to a significant degree. By this we mean, that the increase in free fatty acid does not adversely affect the quality of the foodstuff.

In some aspects of the present invention, the term "without substantially increasing free fatty acids" as used herein means that the amount of free fatty acid in a foodstuff or composition treated with an lipid acyltransferase according to the present invention is less than the amount of free fatty acid produced in the foodstuff or composition when an enzyme other than a lipid acyltransferase according to the present invention had been used, such as for example as compared with the amount of free fatty acid produced when a conventional phospholipase enzyme, e.g. LipopanF® (Novozymes A/S, Denmark), had been used.

The term "*in situ*" as used herein means that the emulsifier(s) and/or the sterol/stanol esters and/or the carbohydrate esters and/or the protein esters and/or the mono- or diglycerides are produced within the foodstuff or fraction of the foodstuff. This contrasts the situation where the emulsifier(s) and/or the sterol/stanol esters and/or the

5   carbohydrate esters and/or the protein esters and/or the mono- or diglycerides are produced separately of the foodstuff and are added as formed products to the foodstuff during preparation of the same. In other words, the term "*in situ*" as used herein means that by the addition of the lipid acyltransferase enzyme according to the present invention to a foodstuff, or to the food ingredients/materials constituting the foodstuff,

10   an emulsifier and/or a sterol ester and/or a stanol ester and/or a carbohydrate ester and/or a protein ester and/or a mono- or diglyceride may be produced from components of the foodstuff. Suitably, the components of the foodstuff may be the substrate(s) for the enzyme. If necessary, the components of the foodstuff may be supplemented by addition of one or more further components which further

15   components may be the same as those present in the foodstuff or may be additional to those components already present in the foodstuff. For the avoidance of doubt, in one embodiment, the method according to the present invention may be a method for the production of an emulsifier and/or a sterol ester and/or a stanol ester and/or a carbohydrate ester and/or a protein ester and/or a mono- or diglyceride *in situ* in a

20   foodstuff and is not a method for preparing an emulsifier and/or a sterol ester and/or a stanol ester (for example is an isolated and/or purified form) for subsequent addition to a foodstuff.

In another embodiument the lipase acyl-transferase may be used during the food
25   processing , but not remain in the foodstuff. For example, the lipase acyl transferase may be immobilized, allowing it to be reused.

Preferably, the lipid acyltransferase according to the present invention is capable of transferring an acyl group from a lipid to a sterol and/or stanol and/or a carbohydrate
30   and/or a protein and/or glycerol when present in a polar environment, preferably in an aqueous environment, preferably a water containing foodstuff. Suitably, the aqueous environment may be an aqueous buffer or may be the aqueous phase in a foodstuff.

The term "aqueous environment" as used herein preferably means an environment which is absent an organic solvent, preferably absent a polar organic solvent, more prefably absent an non-edible organic solvent. In particular, the term "aqueous environment" may refer to an environment to which no exogenous organic solvents, preferably no polar organic solvents, have been added. The term organic solvent as used herein does not encompass food oils, preferably does not encompass food oils that are high in non-polar lipids. In one embodiment the term organic solvent may exclude edible organic solvents, such as ethanol and/or glycerol. Suitably, the aqueous environment according to the present invention may comprise less than 80% by volume organic solvents, less than 70% by volume organic solvents, less than 50% by volume organic solvents, less than 30% by volume organic solvents, more preferably less than 15% by volume organic solvents, more preferably less than 5%. Suitably the foodstuff may comprise between 1 and 5% organic solvent, for example ethanol. However, when the foodstuff comprises such an organic solvent, preferably it is produced endogenously within the foodstuff. That is to say, when the foodstuff comprises such an organic solvent, preferably the organic solvent is not an exogenous organic solvent.

The term "foodstuff" as used herein means a substance which is suitable for human and/or animal consumption.

Suitably, the term "foodstuff" as used herein may mean a foodstuff in a form which is ready for consumption. Alternatively or in addition, however, the term foodstuff as used herein may mean one or more food materials which are used in the preparation of a foodstuff. By way of example only, the term foodstuff encompasses both baked goods produced from dough as well as the dough used in the preparation of said baked goods.

In a preferred aspect the present invention provides a foodstuff as defined above wherein the foodstuff is selected from one or more of the following: eggs, egg-based products, including but not limited to mayonnaise, salad dressings, sauces, ice creams, egg powder, modified egg yolk and products made therefrom; baked goods, including

breads, cakes, sweet dough products, laminated doughs, liquid batters, muffins, doughnuts, biscuits, crackers and cookies; confectionery, including chocolate, candies, caramels, halawa, gums, including sugar free and sugar sweetened gums, bubble gum, soft bubble gum, chewing gum and puddings; frozen products including sorbets,

5 preferably frozen dairy products, including ice cream and ice milk; dairy products, including cheese, butter, milk, coffee cream, whipped cream, custard cream, milk drinks and yoghurts; mousses, whipped vegetable creams, meat products, including processed meat products; edible oils and fats, aerated and non-aerated whipped products, oil-in-water emulsions, water-in-oil emulsions, margarine, shortening and

10 spreads including low fat and very low fat spreads; dressings, mayonnaise, dips, cream based sauces, cream based soups, beverages, spice emulsions, sauces and mayonnaise.

Suitably the foodstuff in accordance with the present invention may be a "fine foods", including cakes, pastry, confectionery, chocolates, fudge and the like.

15

In one aspect the foodstuff in accordance with the present invention may be a dough product or a baked product, such as a bread, a fried product, a snack, cakes, pies, brownies, cookies, noodles, snack items such as crackers, graham crackers, pretzels, and potato chips, and pasta.

20

In a further aspect, the foodstuff in accordance with the present invention may be a plant derived food product such as flours, pre-mixes, oils, fats, cocoa butter, coffee whitener, salad dressings, margarine, spreads, peanut butter, shortenings, ice cream, cooking oils.

25

In another aspect, the foodstuff in accordance with the present invention may be a dairy product, including butter, milk, cream, cheese such as natural, processed, and imitation cheeses in a variety of forms (including shredded, block, slices or grated), cream cheese, ice cream, frozen desserts, yoghurt, yoghurt drinks, butter fat,

30 anhydrous milk fat, other dairy products. The enzyme according to the present invention may improve fat stability in dairy products.

It is particularly advantageous to utilise the present invention in cheese as the production of free fatty acids in cheese is associated with a "soapy" taste. Thus, the use of a lipid acyltransferase in accordance with the present invention advantageously produces cheese without a "soapy" taste.

5

In another aspect, the foodstuff in accordance with the present invention may be a food product containing animal derived ingredients, such as processed meat products, cooking oils, shortenings.

10   In a further aspect, the foodstuff in accordance with the present invention may be a beverage, a fruit, mixed fruit, a vegetable or wine. In some cases the beverage may contain up to 20 g/l of added phytosterols.

In another aspect, the foodstuff in accordance with the present invention may be an
15   animal feed. The animal feed may be enriched with phytosterol and/or phytostanols, preferably with beta-sitisterol/stanol. Suitably, the animal feed may be a poultry feed. When the foodstuff is poultry feed, the present invention may be used to lower the cholesterol content of eggs produced by poultry fed on the foodstuff according to the present invention.

20

In one aspect preferably the foodstuff is selected from one or more of the following: eggs, egg-based products, including mayonnaise, salad dressings, sauces, ice cream, egg powder, modified egg yolk and products made therefrom.

25   Preferably the foodstuff according to the present invention is a water containing foodstuff. Suitably the foodstuff may be comprised of 10-98% water, suitably 14–98%, suitably of 18-98% water, suitably of 20-98%, suitably of 40-98%, suitably of 50-98%, suitably of 70-98%, suitably of 75-98%.

30   For some aspects, preferably the foodstuff in accordance with the present invention is not a pure plant derived oil, such as olive oil, sunflower oil, peanut oil, rapeseed oil for instance. For the avoidance of doubt, in some aspects of the present invention the

foodstuff according to the present invention may comprise an oil, but preferably the foodstuff is not primarily composed of oil or mixtures of oil. For some aspects, preferably the foodstuff comprises less than 95% lipids, preferably less than 90% lipids, preferably less than 85%, preferably less than 80% lipids. Thus, for some

5 aspects of the present invention oil may be a component of the foodstuff, but preferably the foodstuff is not an oil *per se*.

The claims of the present invention are to be construed to include each of the foodstuffs listed above.

10

When it is the case that a carbohydrate ester is produced in accordance with the present invention, the carbohydrate ester is preferably an oligosaccharide ester, a monosaccharide ester or a disaccharide ester.

15 Suitably, the carbohydrate ester when produced in accordance with the present invention may be one or more of the following: glucose ester, fructose ester, anhydrofructose ester, maltose ester, lactose ester, galactose ester, xylose ester, xylooligosaccharide ester, arabinose ester, maltooligosaccharide ester, tagatose ester or sucrose ester.

20

Preferably, the carbohydrate ester when produced in accordance with the present invention is one or more of the following: a carbohydrate mono-ester, a sugar mono-ester, an oligosaccharide mono-ester, a trisaccharide mono-ester, a disaccharide mono-ester, a monosaccharide mono-ester, a glucose mono-ester, a fructose mono-ester,

25 anhydrofructose mono-ester, maltose mono-ester, lactose mono-ester, galactose mono--ester, xylose mono-ester, xylooligosacchride mono-ester, arabinose mono-ester, maltooligosaccharide mono-ester, tagatose mono-ester or sucrose mono-ester.

Preferably, the formation of the carbohydrate ester (if any) in accordance with the

30 present invention is independent of UDP-glucose.

Preferably, the foodstuff according to the present invention does not comprise UDP-glucose, or only comprises UDP-glucose in insignificant amounts.

Suitably, the emulsifier in accordance with the present invention may be for example
5 one or more of the following: a diglyceride, a monoglyceride, such as 1-monoglyceride or a lysolecithin, such as lysophosphatidylcholine for example, a digalactosyl monoglyceride (DGMG). The emulsifier is preferably produced from the lipid acyl donor following removal of one or more acyl groups from said lipid acyl donor. The term lysolecithin as used herein encompasses lysophosphatidylcholine,
10 lysophosphatidylethanolamine, lysophosphatidylinositol, lysophosphatidylserine and lysophosphatidylglycerol

Where one of the emulsifiers is a carbohydrate ester, the second emulsifier may be for example one or more of the following: a diglyceride, a monoglyceride, such as 1-
15 monoglyceride, lysophosphatidylcholine, or digalactosyl monoglyceride (DGMG). The second emulsifier is preferably produced from the lipid acyl donor following removal of one or more acyl groups from said lipid acyl donor. The term lysophosphatidylcholine as used herein is synonymous with the term lysolecithin and these terms may be used herein interchangeably.
20

Preferably the second emulsifier is DGMG. Suitably, the DGMG is produced *in situ* by the removal of an acyl group from DGDG with the transfer of the removed acyl group onto a carbohydrate to form a carbohydrate ester.

25 Where one of the emulsifiers a protein ester and/or a diglyceride and/or a monoglyceride, the second emulsifier may be for example one or more of the following: a diglyceride, a monoglyceride, such as 1-monoglyceride, lysophosphatidylcholine, or digalactosyl monoglyceride (DGMG). The second emulsifier is preferably produced from the lipid acyl donor following removal of one
30 or more acyl groups from said lipid acyl donor. The term lysophosphatidylcholine as used herein is synonymous with the term lysolecithin and these terms may be used herein interchangeably.

In one embodiment the lipid acyl transferase of the invention can be used in a process for the preparation of a foodstuff such as a cooking oil, margarine or spread, whereby the foodstuff naturally contains, or has been supplemented with, glycerol, at least one phospholipid (for example lecithin) and/or glycolipid (for example digalactosyl-diglyceride), and optionally a phytosterol or phytostanol.

When used as a cooking oil or margarine, the foodstuff may have enhanced anti-splattering properties. In addition or alternatively the foodstuff may have one or more beneficial technical properties, for example improved oxidative stability, improved emulsification properties, or health benefits.

In one embodiment the lipid acyl transferase of the invention can be in the preparation of low fat foodstuffs, such as low fat spreads, low fat salad dressings, low fat mayonnaise, low fat margarines etc. In such low fat food products, the fat content is typically reduced by the addition of emulsifiers and additional water compared to the higher fat equivalent.

The lipid acyl transferases used in the compositions and methods of the invention have been found to have unique properties when compared to lipolytic enzymes in that they have a marked preference for transfer of acyl groups from lipids to acceptors other than water, even in the presence of significant water. In a comparison with prior art enzymes, the lipid acyl transferase used in the invention were found to have a high relative transferase activity in the presence of 6% water, 54% water, 73% water, 89% water and approximately 95%. Lipolytic enzymes tested had virtually no significant relative transferase activity at these water concentrations.

The lipase and acyltransferase activity of an enzyme may be evaluated using the following assays. In this way, a lipid acyltransferase having the enzyme characteristics defined herein may be obtained/identified.

Transferase Assay in Buffered Substrate (see Example 12)

Enzymes which function as lipid acyltransferases for use in the compositions and methods of the invention can be routinely identified using the assay taught herein in

5 Example 12. This assay will be hereinafter referred to as the 'Transferase Assay in Buffered Substrate'. In Example 12 the lipid acyltransferase enzyme from *Aeromonas salmonicida* in accordance with the present invention was analysed and compared with a range of lipolytic enzymes not encompassed by the present invention. As can be seen, of the lipolytic enzymes only LIPOPAN® F (Novozymes, Denmark) was found

10 to have any transferase activity and then only a very low level (1.3%).

Enzymes suitable for use in the compositions and methods of the invention can be routinely identified using the Transferase Assay in Buffered Substrate. Using this assay, in which there is a very high water content – approximately 95%, lipid

15 acyltransferases in accordance with the present invention are those which have at least 2% acyltransferase activity (relative transferase activity), preferably at least 5% relative transferase activity, preferably at least 10% relative transferase activity, preferably at least 15%, 20%, 25% 26%, 28%, 30%, 40% 50%, 60% or 75% relative transferase activity. Suitably, the lipid acyltransferase in accordance with the present

20 invention may have less than 28%, less than 30%, preferably less than 40%, 50%, 60%, 70%, 80%, 90% or 100% acyltransferase activity.

Transferase Assay in high water egg yolk (see Example 11)

25 As an alternative to (or in addition to) using the "Transferase Assay in Buffered Substrate" (see above), a lipid acyltransferase for use in accordance with the present invention may be identified using the "Transferase Assay in High Water Egg Yolk" taught in Example 11.

30 In one embodiment, the lipid acyltransferase suitable for use in the methods and/or compositions according to the present invention is one which when tested using the Transferase Assay in High Water Egg Yolk in an egg yolk with 54% water, has up to

100% relative transferase activity. Indeed, experiments in high water egg yolk have shown that at the start of the experiment the initial transferase rate was calculated to be 100% transferase activity, i.e. no hydrolytic activity was observed. In contrast, the lipolytic enzymes used as control, i.e. LIPOPAN® F and phospholipase A2, showed no

5   detectable transferase activity in egg yolk with 54% water, or egg yolk with enriched water content (namely egg yolk with 73% water or 89% water). Preferably the increase in water content does not significantly decrease the percentage acyl transferase activity of a lipid acyltransferase for use in the methods or compositions according to the present invention.

10

In a preferable embodiment, with reference to the Transferase Assay in High Water Egg Yolk, with a water content of 54%, a lipid acyltransferase for use in the present invention will have an initial percentage acyltransferase activity (initial relative transferase activity) measured after 10% consumption of the donor molecule (i.e.

15   phospholipid) of at least 0.1% relative transferase activity, preferably at least 1% relative transferase activity, preferably at least 5% relative transferase activity, preferable at least 10% relative transferase activity, preferably at least 20% relative transferase activity, preferably at least 30% relative transferase activity, preferably at least 40% relative transferase activity, preferably at least 50% relative transferase

20   activity, preferably at least 60%, preferably at least 70%, preferably at least 80%, preferably at least 90%, preferably at least 95%, preferably at least 99%, preferably about 100% acyl transferase activity.

In a preferable embodiment, with reference to the Transferase Assay in High Water

25   Egg Yolk, with a water content of 54%, and measured after 10% consumption of the donor molecule (i.e. phospholipid), the lipid acyltransferase for use in the compositions and methods of the invention has detectable transferase activity, i.e. relative transferase activity of between 0.1 and 100%, preferably at least 1% relative transferase activity, preferably at least 5% relative transferase activity, preferable at

30   least 10% relative transferase activity, preferably at least 20% relative transferase activity, preferably at least 30% relative transferase activity, preferably at least 40% relative transferase activity, preferably at least 45%, 50%, 60%, 70%, 80%, or 90%

relative transferase activity. Suitably, the lipid acyl transferase in accordance with the present invention may have, when using the Transferase Assay in High Water Egg Yolk with 54% water content and measured after 10% consumption of the donor molecule (i.e. phospholipid), a percentage acyl transferase activity (relative transferase activity) of less than 45%, 47%, 50%, 60%, 70%, 80%, 90% or 100%.

5

In a preferable embodiment, with reference to the Transferase Assay in High Water Egg Yolk, with a water content of 73%, measured after 10% consumption of the donor molecule (i.e. phospholipid), the lipid acyltransferase for use in the compositions and methods of the invention has detectable transferase activity, i.e. relative transferase activity of between 0.1 and 100%, preferably at least 1% relative transferase activity, preferably at least 5% relative transferase activity, preferable at least 10% relative transferase activity, preferably at least 20% relative transferase activity, preferably at least 30% relative transferase activity, preferably at least 40% relative transferase activity, preferably at least 45%, 50%, 58%, 60%, 70%, 80%, or 90% relative transferase activity. Suitably, the lipid acyl transferase in accordance with the present invention may have, when using the Transferase Assay in High Water Egg Yolk with 73% water content and measured after 10% consumption of the donor molecule (i.e. phospholipid), a percentage acyl transferase activity (relative transferase activity) of less than 45%, 47%, 50%, 58%, 60%, 70%, 80%, 90% or 100%.

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In a preferable embodiment, with reference to the Transferase Assay in High Water Egg Yolk, with a water content of 89%, and measured after 10% consumption of the donor molecule (i.e. phospholipid), the lipid acyltransferase for use in the compositions and methods of the invention has detectable transferase activity, i.e. relative transferase activity of between 0.1 and 100%, preferably at least 1% relative transferase activity, preferably at least 5% relative transferase activity, preferable at least 10% relative transferase activity, preferably at least 20% relative transferase activity, preferably at least 30% relative transferase activity, preferably at least 40% relative transferase activity, preferably at least 45%, 50%, 60%, 70%, 80%, or 90% relative transferase activity. Suitably, the lipid acyl transferase in accordance with the present invention may have, when using the Transferase Assay in High Water Egg

25

30

Yolk with 89% water content and measured after 10% consumption of the donor molecule (i.e. phospholipid), a percentage acyl transferase activity (relative transferase activity) of less than 45%, 47%, 50%, 60%, 70%, 80%, 90% or 100%.

5   In a preferable embodiment, with reference to the Transferase Assay in High Water Egg Yolk, a lipid acyltransferase for use in the compositions and methods of the invention has significant relative transferase activity (i.e. at least 0.1% at both water contents), and has an equivalent relative transferase activity in egg yolk with a water content of 54% as in an egg yolk with a water content of 73%, when measured after
10   10% consumption of the donor molecule (i.e. phospholipid).

In a preferable embodiment, with reference to the Transferase Assay in High Water Egg Yolk, a lipid acyltransferase for use in the compositions and methods of the invention has significant relative transferase activity (i.e. at least 0.1% at both water
15   contents), and has an equivalent relative transferase activity in egg yolk with a water content of 54% as in an egg yolk with a water content of 89%, when measured after 10% consumption of the donor molecule (i.e. phospholipid).

In a preferable embodiment, with reference to the Transferase Assay in High Water
20   Egg Yolk, a lipid acyltransferase for use in the compositions and methods of the invention has significant relative transferase activity (i.e. at least 0.1% at both water contents), and has an equivalent relative transferase activity in egg yolk with a water content of 73% as in an egg yolk with a water content of 89%, when measured after 10% consumption of the donor molecule (i.e. phospholipid).
25

The term "equivalent relative transferase activity" as referred to herein means that the enzyme has a relative transferase activity (% acyltransferase activity) which is at least 10% lower, preferably at least 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90% lower, in the egg yolk with the higher water content compared with that in the egg yolk with
30   the lower water content.

Transferase Assay in a Low Water Environment

As an alternative to (or in addition to) using the "Transferase Assay in High Water Egg Yolk" and/or the "Transferase Assay in Buffered Substrate", lipid acyltransferases for use in accordance with the present invention may be identified using the "Transferase Assay in a Low Water Environment".

In order to determine if an enzyme is a lipid acyltransferase according to the present invention, one may carry out a "Transferase Assay in a Low Water Environment", namely in an oily environment with 6% water as taught in Example 22. This example illustrates that in an oily environment with 6% water content the lipid acyltransferase of the invention has a high relative transferase activity, where the prior art lipolytic enzymes have hydrolytic activity.

In one embodiment, the lipid acyltransferase suitable for use in the methods and/or compositions according to the present invention is one which when tested using the "Transferase Assay in a Low Water Environment", measured after a time period selected from 30, 20 or 120 minutes, has a relative transferase activity of at least 1%, preferably at least 2%, preferably at least 5%, preferably at least 10%, preferably at least 20%, preferably at least 30%, preferably at least 40%, preferably at least 50%, preferably at least 60%, preferably at least 70%, preferably at least 75%. Suitably, the lipid acyl transferase in accordance with the present invention may have less than 30%, 40%, 50%, 60%, 70%, or 80% activity when measured after a time period of 10, 20, 30 or 120 minutes using the "Transferase Assay in a Low Water Environment".

As described above, the lipase acyltransferase of the invention can be identified using either the "Transferase Assay in Buffered Substrate" or in the "Transferase Assay in Low Water Environment" using cholesterol as the acyl acceptor. Of course, the skilled person would be readily aware that, with obvious amendments to the analytical methods the 'Transferase Assay in Buffered Substrate' or the 'Transferase Assay in Low Water Environment may be used to determine the lipid acyltransferase activity for any lipid acyl donor or any acyl acceptor combination. The skilled person would,

if necessary, simply replace the acyl donor substrate (e.g. phospholipid) with an alternative acyl donor substrate (e.g. glycolipid, triacylglyceride) and/or replace the acyl acceptor (e.g. cholesterol) with an alternative acyl acceptor substrate (e.g. a carbohydrate, a protein, another sterol, a stanol or glycerol).

5

The term "high water" as used herein means any substrate or foodstuff with more than 2% water content, preferably more than 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90%.

10   The term "low water" as used herein means any substrate or foodstuff with less than 6% water content, preferably less than 5%, 4%, 3%, 2%, 1% or 0.5%.

Preferably the method and/or use according to the present invention may be carried out, for example, in foodstuff at a temperature of 15-60°C, preferably at a temperature

15   of 20-60°C, preferably 20-50°C, preferably 20-45°C, preferably 20-40°C. For some aspects, for example in dough, preferably the temperature of the food during which the acyltransferase reaction takes place is between 20 and 40°C. For other aspects, for example with regard to dairy products, such as cheese, the temperature of the food may suitably be between 30°C and 60°C. In yet other aspects, for example with regard

20   to mayonnaise, the temperature of the food may suitably be between 20 and 40°C, more preferably between 25 and 30°C.

Preferably, the emulsifier produced according to the present invention comprises less than 5 wt % of the foodstuff.

25

Preferably, the emulsifier produced according to the present invention comprises from 0.01 to 4 wt % of the foodstuff.

Preferably, the emulsifier produced according to the present invention comprises from

30   0.01 to 2 wt % of the foodstuff.

Preferably, the emulsifier produced according to the present invention comprises from 0.01 to 1 wt % of the foodstuff.

Preferably, the emulsifier produced according to the present invention comprises from 0.01 to 0.5 wt % of the foodstuff.

Preferably, the emulsifier produced according to the present invention comprises from 0.01 to 0.3 wt % of the foodstuff.

Suitably, the method according to the present invention includes inactivating or denaturing the enzyme to provide a foodstuff comprising the enzyme in an inactive or denatured form. Suitably the enzyme may be denatured by either baking or by pasteurisation.

The present invention may further encompass the use of a lipid acyltransferase as defined herein in food and/or feed enzyme compositions, and may encompass food and/or feed enzyme compositions comprising a lipid acyltransferase as defined herein. Such compositions may contain one or more further enzymes, such as those listed herein. Alternatively, the enzyme composition of the invention may be used in combination with other food ingredients/additives, such as those listed herein, including other enzyme compositions. By formulation of the lipid acyl transferase of the invention within a food and/or feed composition, the enzyme can be stabilised to allow for prolonged storage (under suitable conditions) prior to use in food and/or feed production. In addition the enzyme composition of the present invention provides the enzyme in a suitable form for safe use for the 'in situ' application in the preparation of foodstuffs and/or feedstuffs, or ingredients for use in food and/or feed preparation. Such compositions may be in either liquid, semi-liquid or solid/granular form.

Food enzymes are sold as stabilized liquid concentrates or as particulate solids. Formulation into food enzyme composition minimizes losses in enzymatic activity during transport, storage, and use. Enzymes are often exposed to humid, hot, or oxidative environments in food and beverage processing. Formulations enhance

stability by counteracting the primary forces of deactivation: denaturation, catalytic-site deactivation, and proteololysis. Denaturation occurs by physical unfolding of an enzyme's tertiary protein structure under thermal or chemical stress. Once an enzyme begins to unfold it becomes dramatically more vulnerable to deactivation and

5 proteolysis. To minimize unfolding, the formulator can alter the protein's environment so as to induce a compact protein structure; this is done most effectively by "preferential exclusion" of water from the protein surface by adding water-associating compounds such as sugars, polyhydric alcohols, and lyotropic salts. The best ways to combat active site inactivation are to ensure sufficient levels of any required cofactors,

10 to add reversible inhibitors, and to exclude oxidizing or reactive species from the formulation.

Besides enzymatic stability, a formulation should meet several key secondary requirements, including preservation against microbial contamination, avoidance of

15 physical precipitation or haze formation, minimizing the formation of sensitizing dusts or aerosols, and the optimization of esthetic criteria such as color and odor. Many of these problems are best addressed by focusing as far "upstream" as possible, including the choice of raw materials in the fermentation or enzyme recovery process. Downstream operations such as diafiltration, adsorption, chromatography,

20 crystallization, and extraction can be used to remove impurities responsible for color, odor, and precipitation The risk of physical precipitation is minimized by formulating near the isoelectric point of the enzyme with hydrophilic solvents such as glycerol or propylene glycol. One can effectively also add moderate levels of solvating salts to avoid either salting-out or "reverse salting-in". To prevent microbial contamination,

25 one can use a combination of filtration, acidification, and the minimization of free water; biocides can be effective, but the range of acceptable chemicals for controlling or killing microbes is increasingly circumscribed by health and safety regulations.

Two processes producing the most attrition-resistant granules to date are high-shear

30 granulation and fluidized-bed spray coating, see for example T. Becker: "Separation and Purification Processes for Recovery of Industrial Enzymes" in R. K. Singh, S. S. H. Rizvi (eds.): *Bioseparation Processes in Foods*, Marcel Dekker, New York, pp.

427 – 445. These processes use various binders, coatings, and particle morphologies to produce nonfriable particles which still protect enzymes during storage but allow for their ready release in solution during use.

5   Food enzyme compositions containing the lipid acyl transferase of the invention may be made using standard formulation techniques, such as spray drying or liquid formulation.

The lipid acyl-transferase of the invention can be expressed in any suitable expression

10   host. For example the lipid acyltransferase of the invention may be expressed in *Bacillus subtilis* and may be purified by ultrafiltration and/or by precipitation in ethanol and/or centrifugation, and may be subsequently spray dried using starch (maltodextrin) as carrier for the enzyme. The spray dried enzyme may be standardized to specified PLU activity by adding further carrier in powder form. The techniques

15   involved are well established and routine in the art.

Alternatively, lipid acyltransferase for use in accordance with the present invention, for example the heterologously produced lipid acyl-transferase of the invention, once purified, may be stabilised in a suitable liquid formulation, such as those based on

20   glycerol. Other methods of making stabilised enzyme formulations are described in EP 0 770 037 and EP 0 702 712).

The acyl transferase in powder form can also be used in combination with other enzymes as listed herein, for the production of enzyme compositions with defined

25   activity according to the product specification.

Typically the dosage of the food enzyme formulation is between 10g and 1000g per 1000kg of foodstuff, preferably 50-200g per 1000kg of foodstuff, preferably, 75-125gm per 1000kg of foodstuff.

30

Preferably the enzyme according to the present invention is present in an inactive form or in a denatured form in the foodstuff.

In one embodiment, the enzyme according to the present invention is preferably not immobilised, in particular is not immobilised on a solid support.

5   In an alternative embodiment, the enzyme may be immobilised.

mmobilised lipid acyl transferase can be prepared using immobilisation techniques known in the art. There are numerous methods of preparing immobilised enzymes, which will be apparent to a person skilled in the art (for example the techniques

10   referred to in EP 0 746 608; or Balcao VM, Paiva AL, Malcata FX., Enzyme Microb Technol. 1996 May 1;18(6):392-416; or Reetz MT, Jaeger KE.Chem Phys Lipids. 1998 Jun;93(1-2):3-14; or Bornscheuer UT, Bessler C, Srinivas R, Krishna SH.Trends Biotechnol. 2002 Oct; 20(10):433-7 (each of which is incorporated herein by reference).

15

In one embodiment, the foodstuff of the invention may contain food ingredients, which have been prepared using immobilised lipid acyltransferase, but do not contain the lipid acyltransferase in the food ingredient or foodstuff. For example the foodstuff may contain one or more of the following: an emulsifier, more than one emulsifier,

20   one or more flavouring agents, one or more textural enhancers and/or one or more sterol esters, such as phytosterol esters or phytostanol esters.

The enzyme according to the present invention may be used with one or more other suitable food grade enzymes. Thus, it is within the scope of the present invention that,

25   in addition to the enzyme of the invention, at least one further enzyme is added to the foodstuff. Such further enzymes include starch degrading enzymes such as endo- or exoamylases, pullulanases, debranching enzymes, hemicellulases including xylanases, cellulases, oxidoreductases, e.g. glucose oxidase or a carbohydrate oxidase such as one which oxidises maltose, for example hexose oxidase (HOX), lipases, phospholipases

30   and hexose oxidase, and proteases.

In one preferred embodiment the lipid acyltransferase is used in combination with a lipase having one or more of the following lipase activities: glycolipase activity (E.C. 3.1.1.26, triacylglycerol lipase activity (E.C. 3.1.1.3), phospholipase A2 activity (E.C. 3.1.1.4) or phospholipase A1 activity (E.C. 3.1.1.32). . Suitably, lipase enzymes are

5   well know within the art and include by way of example the following lipases: LIPOPAN® F and/or LIPOPAN® ULTRA (Novozymes A/S, Denmark), phospholipase A2 (e.g. phospholipase A2 from LIPOMOD™ 22L from Biocatalysts, LIPOMAX™ from Genecor), LIPOLASE® (Novozymes A/S, Denmark), the lipases taught in WO03/97835, EP 0 977 869 or EP 1 193 314. This combination of a lipid acyl

10   transferase as defined herein and a lipase may be particularly preferred in dough or baked products or in fine food products such as cakes and confectionary.

The use of lipases in combination with the enzyme of the invention may be particularly advantageous in instances where some accumulation of free fatty acids

15   maybe desirable, for example in cheese where the free fatty acids can impart a desirable flavour, or in the preparation of fine foods. The person skilled in the art will be able to combine proportions of lipolytic enzymes, for example LIPOPAN® F and/or LIPOPAN® ULTRA (Novozymes A/S, Denmark), phospholipase A2 (e.g. phospholipase A2 from LIPOMOD™ 22L from Biocatalysts, LIPOMAX™ from

20   Genecor), LIPOLASE® (Novozymes A/S, Denmark), the lipases taught in WO03/97835, EP 0 977 869 or EP 1 193, 314 and the lipid acyltransferase of the present invention to provide the desired ratio of hydrolytic to transferase activity which results in a preferred technical effect or combination of technical effects in the foodstuff (such as those listed herein under 'Technical Effects').

25

Traditionally the cake industry uses cake improvers for the production of cakes and to secure high quality cakes in terms of taste, structure, eating quality and appearance. These cake improvers are normally based on emulsifiers spray dried on a carrier like starch and malto dextrin. Some cake improvers are also in a gel form based on

30   emulsifiers, sugars and water. These cake improvers are very important for the cake industry in order to produce cake of high quality. Cake improvers however contain

emulsifiers and other "non-natural" ingredients with an E-number. Because of demand for the consumers to reduce the numbers of E-numbers, the cake industry has asked for alternative ways to produce cakes of high quality without using emulsifiers.

5   An alternative way to produce cake is to use an enzyme, i.e. the lipid acyltransferase defined herein or an enzyme composition according to the present invention.

The lipid acyltransferase as defined herein and/or the food enzyme composition of the present invention may be used in the preparations of a fine food, such as a cake. In
10   such instances, the following constituents may be formed in the fine food:
   i)   sugar esters and lysolecithin (from the carbohydrate in the cake recipe and the lecithin in egg which also form part of the cake recipe); and/or
   ii) acylated peptides and lysolecithin (by transferring a fatty acid from lecithin to a protein or peptide during formation of protein-fatty acid condensates,
15   which are known to be highly efficient emulsifiers (Herstellung und Anvendungmöglichkeiten von Eiweiss-Fettsäurekondensaten. Andreas Sander, Eberhard Eilers, Andrea Heilemann, Edith von Kreis.Fett/lipid 99 (1997) Nr. 4, 115-120).

20   It is considered that in the production of some fine foods, particularly high fat fine foods, such as cakes, it may be desirable to have some accumulation of fatty acids. Therefore the combination of the use of lipolytic enzymes and the lipid acyl transferase as defined herein may be particularly beneficial for production of high fat fine foods. Alternatively, additional free fatty acids may be selected and used in
25   combination with the lipid acyl transferase.

The foodstuff according to the present invention may suitably comprise one or more of the following additives:
soy   protein material; carotenoids, flavenoids, antioxidant and phytochemical
30   (especially anthocyanonide, carotenoid, bioflavinoid, glutathione, catechin, isoflavone, lycopene, ginsenoside, pycnogenol, alkaloid, pygeum phytosterol, sulphoraphone, resveretol, grape seed extract or food containing stanol esters), vitamin (especially

vitamin C, vitamin A, vitamin B3, vitamin D, vitamin E, thiamine, riboflavin, niacin, pyridoxine, cyanocobalamin, folic acid, biotin, pantothenic acid or vitamin K), minerals (especially calcium, iodine, magnesium, zinc, iron, selenium, manganese, chromium, copper, cobalt, molybdenum or phosphorus), fatty acid (especially gamma-
5   linoleic acid, ucospentaenoic acid or decosahexaenoic acid), oil (especially borage oil, high carotenoid canola oil or flax seed oil), amino acid (especially tryptophan, lysine, methionine, phenylalanine, threonine, valine, leucine, isoleucine, alanine, arginine, aspartic acid, cystine, cysteine, glutamic acid, glutamine, glycine, histidine, proline, hydroxyproline, serine, taurine or tyrosine), enzyme (especially bromelain, papain,
10   amylase, cellulase or coenzyme Q), lignin, stanol ester or friendly bacteria (especially Lactobacillus   acidophilus,   Lactobacillus   bulgaricus,   Lactobacillus   bifidus, Lactobacillus plantarum or Streptococcus faecium), folic acid, and soluble fibre.

TECHNICAL EFFECT
15

Surprisingly   lipid   acyltransferases   have   significant   acyltransferase   activity   in foodstuffs.   This   activity   has   surprising   beneficial   applications   in   methods   of preparing foodstuffs.

20   The   present   invention   is   predicated   upon   the   surprising   finding   that   the   lipid acyltransferases   according   to   the   present   invention   can   perform   carbohydrate-esterification via alcoholosis, i.e. acyl transfer from a lipid, in a foodstuff with a significant water content. Prior art suggests that such enzymes if they would function at all in this manner would only function in a solvent environment (i.e. in
25   environments with low or no water content).

The present invention may provide one or more of the following unexpected technical effects in egg products, particularly mayonnaise: an improved heat stability during pasteurisation; improved organoleptic properties, an improved consistency.
30

The present invention may provide one or more of the following unexpected technical effects in dough and/or baked products: an improved specific volume of either the

dough or the baked products (for example of bread and/or of cake); an improved dough stability; an improved crust score (for example a thinner and/or crispier bread crust), an improved crumb score (for example a more homogenous crumb distribution and/or a finer crumb structure and/or a softer crumb); an improved appearance (for example a smooth surface without blisters or holes or substantially without blisters or holes); a reduced staling; an enhanced softness; an improved odour; an improved taste.

The present invention may provide a beneficial effect from formation of highly surface-active materials in a foodstuff without formation of substantial amount of free fatty acids, which reduce the ability of the foodstuff to oxidize upon storage, because free fatty acids are more prone to oxidation than the corresponding fatty acid esters.

Suitably, the present invention may provide one or more of the following unexpected technical effects in a foodstuff: an improved appearance, an improved mouthfeel, an improved stability, in particular an improved thermal stability, an improved taste, an improved softness, an improved resilience, an improved emulsification.

Suitably, the present invention may provide one or more of the following unexpected technical effects in dairy products, such as ice cream for example: an improved mouthfeel (preferably a more creamy mouthfeel); an improved taste; an improved meltdown.

Suitably, the present invention may provide one or more of the following unexpected technical effects in egg or in egg products: improved stability of emulsion; thermal stability of emulsion; improved flavour; reduced mal-odour; improved thickening properties, improved consistency.

Specific technical effects associated with the use of a lipid acyltransferase as defined herein in the preparation of a foodstuff are listed in the table below:

	Foodstuff	Effect
1	Bread, Muffins and	Strengthens dough and increases mechanical

	Doughnuts	resistance and increases water absorption capacity. Increases volume of bakery products and maintains softness of crumb
2	Frozen dough	Prevents spoiling during refrigeration
3	Sponge cake	Makes good cake volume and a uniform soft texture
4	Biscuit, cracker and cookie	Makes stable emulsions of fat and prevents stickiness to the machine. Prevents blooming of high fat products
5	Batter and breading	Improves texture of fried products.
6	Noodles	Prevents dough from sticking to the machine. Increases water content, and decreases cooking loss
7	Instant noodles	Prevent noodles form adhering to each other
8	Pasta	Dough conditioner prevents adhesion on cooking.
9	Custard cream	Makes starch paste with a smooth and creamy texture, and prevents dehydration.
10	Coffee whitener	Prevent oil and water separation
11	Whipping cream	Provides stable emulsion
12	Chocolate	Prevents or reduced blooming
13	Caramel, candy and nougat	Improves emulsification of molten sugar and oil. Prevents separation of oil.
14	Processed meat, sausages	Improves water holding capacity of sausages and pressed ham, and prevents separation of oil phase of pastes and pâté.

Suitably, the present invention may provide one or more of the following unexpected technical effects in cheese: a decrease in the oiling-off effect in cheese; an increase in cheese yield; an improvement in flavour; a reduced mal-odour; a reduced "soapy"

5   taste.

In food production, in particular cheese production, the use of the lipid acyltransferase in accordance with the present invention provides a significant advantage in the ability to recover soluble proteins from dairy products. For example, in cheese production

10   nearly 20% of all milk protein is removed in the whey (i.e. the watery part of the milk that remains after the formation of curds). The whey comprises the soluble milk proteins, whereas the hydrophobic proteins are maintained in the curd. By use of the lipid acyltransferase in accordance with the present invention it is possible to transfer an acyl group from a lipid (preferably from a glycolipid or a phospholipid), to a

protein (in particular to a whey protein such as lactoglobulin) to from a protein fatty acid condensate. Thus, producing a product which is more hydrophobic and which will stay in the curd rather than being eluted in the whey. In this way, more of the milk protein can be maintained in the final foodstuff, i.e. the final dairy product such 5 as the cheese.

In one aspect, the present invention is based in part on the realisation that yields of foods – such as cheese - may be improved by the use of a lipid acyl transferase. In addition or alternatively, the flavour, texture, oxidative stability and/or shelf life of the 10 food may be improved. In addition or alternatively, the food may have a reduced cholesterol level or enhanced content of phytosterol/stanol esters.

Without wishing to be bound to a particular theory it is considered that the increase in yield may be the result of the transesterification of whey proteins and peptides, 15 resulting in significant increase in the hydrophobicity of the whey proteins and precipitation of the acylated whey proteins in the cheese curd.

In biological systems, for example, the deposition of membrane bound proteins and enzymes are achieved by two different mechanisms. The membrane bound proteins 20 either possess a number of membrane-spanning or hydrophobic domains, or they have alternatively a fatty acid linked to the polypeptide chain. The fatty acids have normally a chain length of 14 or 16 carbon atoms. The fatty acids are covalently linked to the polypeptide chain at 3 different position, the N-terminal amino acid as an amide-bond, a cysteine residue as a thioester linkage, or a serine or threonine amino acid as an ester 25 linkage. Only one fatty acid per polypeptide molecule is necessary to incorporate the protein into the cell membrane.

When a fatty acid is covalently linked to a non-membrane protein, the physical and functional properties will change drastically. WO97/14713 describes the transformed 30 soy and gluten proteins into acyl derivatives by treatment with a lipase from *Mucor miehei* (Lipozyme™, Novozymes), and a fatty acid in organic solvent. The lipid acyl

transferase according to the present invention may be used in the production of acylated proteins is a low or high water environment.

We note that acylated proteins form amphiphilic complexes that can be used for a number of cosmetic products. The acylated protein can form gels, bind water by retaining moisture, have emulsifying properties and is very active in the interphase between water and lipid.

Thus, the present invention may in one aspect provide a cosmetic composition comprising a lipid acyl transferase as defined herein.

In addition, the present invention may provide the use of an acyltransferase as defined herein to produce a cosmetic composition.

In a further aspect, the present invention provides a method of *in situ* production of a protein ester in a cosmetic composition, wherein the method comprises the step of adding to the cosmetic composition (or components thereof) a lipid acyltransferase as defined herein.

Many food proteins are soluble in aqueous solutions and are therefore suitable for *in situ* modification by the lipase acyl transferase. In the cheese production, β-lactoglobulin is lost to the whey fraction. After acylation with a lipase acyl transferase, or a lipase acyl transferase variant, initial results indicate that b-lactoglobulin may however, be deposited in the casein micelle surface during rennet coagulation. β-lactoglobulin has three potential acylation sites (serine residues) on three surface loops. Milk contains sufficient amounts of lecithin, a suitable substrate for a lipid acyl transferase enzyme to acylate the β-lactoglobulin. The lysolecithin formed may have an additional emulsifying effect.

The improvements observed with lipid acyltransferase according to the present invention are in comparison to when lipolytic enzymes without acyltransferase activity, such as triacylglycerol lipases and phospholipases, are used.

ADVANTAGES .

The generation of an emulsifier and a sterol/stanol ester *in situ* from at least one
5 constituent of the food material, means that the food material will contain at least one
less additive material. This is advantageous because of the improvement in the ease of
production. For example, no further processing or addition of ingredients or addition
of emulsifiers may be required. Moreover, the foodstuff may contain less "additives".
The reduction or elimination of "additives" is desirable to consumers and inclusion of
10 additives often must be declared to the consumer in the ingredients listing on the
foodstuff. Thus, the present invention is further advantageous.

An advantage of the present invention may be the production *in situ* of an emulsifier in
a foodstuff without a detrimental increase in the free fatty acid content of the foodstuff.
15

. The generation of two emulsifiers and/or a carbohydrate ester *in situ* from at least one
constituent of the food material, means that the food material will contain at least one
less additive material.

20 In addition, when the lipid acyltransferase acts on a glycolipid it is possible to
advantageously produce the emulsifier DGMG *in situ* without a detrimental increase in
the free fatty acid content of the foodstuff. Thus, reducing detrimental effects
attributed to an increase in free fatty acids, including but not limited to a reduction in
"soapy" taste in cheese, prevention of overdosing in dough and dough baked
25 properties.

For some aspects, an advantage of the present invention is the reduction in free
cholesterol levels in the foodstuff.

30 For other aspect, an advantage of the present invention is the increase in stanol and/or
sterol esters in the foodstuff. Some sterol/stanol esters may be effective flavourants
and/or texturisers. Thus, the present invention may not only results in the *in situ*

production of an emulsifier in a foodstuff, but also the *in situ* production of a flavourant and/or a texturiser. Some sterol/stanol esters are known to reduce blood serum cholesterol and/or low density lipoproteins when consumed in a foodstuff. Thus, the present invention may be used to prepare a foodstuff with increased levels of sterol

5   esters and/or stanol esters.

For some aspects, particularly when the enzyme according to the present invention is used in egg based products, an advantage is the removal of unwanted free carbohydrates.

10

Also advantageously the emulsification properties of the foodstuff are enhanced, leading to improved appearance and/or handling properties and/or structure and/or consistency and/or heat stability without a negative impact on taste.

15   In addition, for some embodiments advantageously the effect of "overdosing" observed when using lipases *per se*, is effectively overcome by the addition of an enzyme in accordance with the present invention. This is due at least in part to the fact that free fatty acids are not produced or only produced to an insignificant degree when using the enzyme according to the present invention.

20

ISOLATED

In one aspect, preferably the polypeptide or protein for use in the present invention is in an isolated form. The term "isolated" means that the sequence is at least

25   substantially free from at least one other component with which the sequence is naturally associated in nature and as found in nature.

PURIFIED

30   In one aspect, preferably the polypeptide or protein for use in the present invention is in a purified form. The term "purified" means that the sequence is in a relatively pure

state – e.g. at least about 51% pure, or at least about 75%, or at least about 80%, or at least about 90% pure, or at least about 95% pure or at least about 98% pure.

CLONING A NUCLEOTIDE SEQUENCE ENCODING A POLYPEPTIDE
5   ACCORDING TO THE PRESENT INVENTION

A nucleotide sequence encoding either a polypeptide which has the specific properties as defined herein or a polypeptide which is suitable for modification may be isolated from any cell or organism producing said polypeptide. Various methods are well
10   known within the art for the isolation of nucleotide sequences.

For example, a genomic DNA and/or cDNA library may be constructed using chromosomal DNA or messenger RNA from the organism producing the polypeptide. If the amino acid sequence of the polypeptide is known, labelled oligonucleotide
15   probes may be synthesised and used to identify polypeptide-encoding clones from the genomic library prepared from the organism. Alternatively, a labelled oligonucleotide probe containing sequences homologous to another known polypeptide gene could be used to identify polypeptide-encoding clones. In the latter case, hybridisation and washing conditions of lower stringency are used.
20

Alternatively, polypeptide-encoding clones could be identified by inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming enzyme-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing an enzyme inhibited by the polypeptide,
25   thereby allowing clones expressing the polypeptide to be identified.

In a yet further alternative, the nucleotide sequence encoding the polypeptide may be prepared synthetically by established standard methods, e.g. the phosphoroamidite method described by Beucage S.L. et al (1981) Tetrahedron Letters 22, p 1859-1869,
30   or the method described by Matthes et al (1984) EMBO J. 3, p 801-805. In the

phosphoroamidite method, oligonucleotides are synthesised, e.g. in an automatic DNA synthesiser, purified, annealed, ligated and cloned in appropriate vectors.

The nucleotide sequence may be of mixed genomic and synthetic origin, mixed
5   synthetic and cDNA origin, or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate) in accordance with standard techniques. Each ligated fragment corresponds to various parts of the entire nucleotide sequence. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or in
10   Saiki R K et al (Science (1988) 239, pp 487-491).

NUCLEOTIDE SEQUENCES

15   The present invention also encompasses nucleotide sequences encoding polypeptides having the specific properties as defined herein. The term "nucleotide sequence" as used herein refers to an oligonucleotide sequence or polynucleotide sequence, and variant, homologues, fragments and derivatives thereof (such as portions thereof). The nucleotide sequence may be of genomic or synthetic or recombinant origin, which may be double-
20   stranded or single-stranded whether representing the sense or antisense strand.

The term "nucleotide sequence" in relation to the present invention includes genomic DNA, cDNA, synthetic DNA, and RNA. Preferably it means DNA, more preferably cDNA for the coding sequence.
25
In a preferred embodiment, the nucleotide sequence per se encoding a polypeptide having the specific properties as defined herein does not cover the native nucleotide sequence in its natural environment when it is linked to its naturally associated sequence(s) that is/are also in its/their natural environment. For ease of reference, we shall call this preferred
30   embodiment the "non-native nucleotide sequence". In this regard, the term "native nucleotide sequence" means an entire nucleotide sequence that is in its native environment and when operatively linked to an entire promoter with which it is naturally

associated, which promoter is also in its native environment. Thus, the polypeptide of the present invention can be expressed by a nucleotide sequence in its native organism but wherein the nucleotide sequence is not under the control of the promoter with which it is naturally associated within that organism.

Preferably the polypeptide is not a native polypeptide. In this regard, the term "native polypeptide" means an entire polypeptide that is in its native environment and when it has been expressed by its native nucleotide sequence.

Typically, the nucleotide sequence encoding polypeptides having the specific properties as defined herein is prepared using recombinant DNA techniques (i.e. recombinant DNA). However, in an alternative embodiment of the invention, the nucleotide sequence could be synthesised, in whole or in part, using chemical methods well known in the art (see Caruthers MH *et al* (1980) Nuc Acids Res Symp Ser 215-23 and Horn T *et al* (1980) Nuc Acids Res Symp Ser 225-232).

MOLECULAR EVOLUTION

Once an enzyme-encoding nucleotide sequence has been isolated, or a putative enzyme-encoding nucleotide sequence has been identified, it may be desirable to modify the selected nucleotide sequence, for example it may be desirable to mutate the sequence in order to prepare an enzyme in accordance with the present invention.

Mutations may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites.

A suitable method is disclosed in Morinaga *et al* (Biotechnology (1984) 2, p646-649). Another method of introducing mutations into enzyme-encoding nucleotide sequences is described in Nelson and Long (Analytical Biochemistry (1989), 180, p 147-151).

Instead of site directed mutagenesis, such as described above, one can introduce mutations randomly for instance using a commercial kit such as the GeneMorph PCR

mutagenesis kit from Stratagene, or the Diversify PCR random mutagenesis kit from Clontech. EP 0 583 265 refers to methods of optimising PCR based mutagenesis, which can also be combined with the use of mutagenic DNA analogues such as those described in EP 0 866 796. Error prone PCR technologies are suitable for the

5 production of variants of lipid acyl transferases with preferred characterisitics. WO0206457 refers to molecular evolution of lipases.

A third method to obtain novel sequences is to fragment non-identical nucleotide sequences, either by using any number of restriction enzymes or an enzyme such as

10 Dnase I, and reassembling full nucleotide sequences coding for functional proteins. Alternatively one can use one or multiple non-identical nucleotide sequences and introduce mutations during the reassembly of the full nucleotide sequence. DNA shuffling and family shuffling technologies are suitable for the production of variants of lipid acyl transferases with preferred characteristics. Suitable methods for

15 performing 'shuffling' can be found in EP0 752 008, EP1 138 763, EP1 103 606. Shuffling can also be combined with other forms of DNA mutagenesis as described in US 6,180,406 and WO 01/34835.

Thus, it is possible to produce numerous site directed or random mutations into a

20 nucleotide sequence, either *in vivo* or *in vitro*, and to subsequently screen for improved functionality of the encoded polypeptide by various means. Using in silico and exo mediated recombination methods (see WO 00/58517, US 6,344,328, US 6,361,974), for example, molecular evolution can be performed where the variant produced retains very low homology to known enzymes or proteins. Such variants thereby obtained

25 may have significant structural analogy to known transferase enzymes, but have very low amino acid sequence homology.

As a non-limiting example, In addition, mutations or natural variants of a polynucleotide sequence can be recombined with either the wild type or other

30 mutations or natural variants to produce new variants. Such new variants can also be screened for improved functionality of the encoded polypeptide.

The application of the above-mentioned and similar molecular evolution methods allows the identification and selection of variants of the enzymes of the present invention which have preferred characteristics without any prior knowledge of protein structure or function, and allows the production of non-predictable but beneficial

5   mutations or variants. There are numerous examples of the application of molecular evolution in the art for the optimisation or alteration of enzyme activity, such examples include, but are not limited to one or more of the following: optimised expression and/or activity in a host cell or in vitro, increased enzymatic activity, altered substrate and/or product specificity, increased or decreased enzymatic or structural stability,

10 altered enzymatic activity/specificity in preferred environmental conditions, e.g. temperature, pH, substrate

As will be apparent to a person skilled in the art, using molecular evolution tools an enzyme may be altered to improve the functionality of the enzyme.

15

Suitably, the lipid acyltransferase used in the invention may be a variant, i.e. may contain at least one amino acid substitution, deletion or addition, when compared to a parental enzyme. Variant enzymes retain at least 1%, 2%, 3%, 5%, 10%, 15%, 20%, 30%, 40%, 50 %, 60%, 70%, 80%, 90%, 95%, 97%, 99% homology with the parent

20 enzyme. Suitable parent enzymes may include any enzyme with esterase or lipase activity. Preferably, the parent enzyme aligns to the pfam00657 consensus sequence.

In a preferable embodiment a variant lipid acyltransferase enzyme retains or incorporates at least one or more of the pfam00657 consensus sequence amino acid

25 residues found in the GDSx, GANDY and HPT blocks.

Enzymes, such as lipases with no or low lipid acyltransferase activity in an aqueous environment may be mutated using molecular evolution tools to introduce or enhance the transferase activity, thereby producing a lipid acyltransferase enzyme with

30 significant transferase activity suitable for use in the compositions and methods of the present invention.

Suitably, the lipid acyltransferase for use in the invention may be a variant with enhanced enzyme activity on polar lipids, preferably phospholipids and/or glycolipids when compared to the parent enzyme. Preferably, such variants also have low or no activity on lyso polar lipids. The enhanced activity on polar lipids, phospholipids
5   and/or glycolipids may be the result of hydrolysis and/or transferase activity or a combination of both.

Variant lipid acyltransferases for use in the invention may have decreased activity on triglycerides, and/or monoglycerides and/or diglycerides compared with the parent
10   enzyme.

Suitably the variant enzyme may have no activity on triglycerides and/or monoglycerides and/or diglycerides.

15   Alternatively, the variant enzyme for use in the invention may have increased activity on triglycerides, and/or may also have increased activity on one or more of the following, polar lipids, phospholipids, lecithin, phosphatidyl cholin, glycolipids, digalactosyl monoglyceride, monogalactosyl monoglyceride.

20   AMINO ACID SEQUENCES

The present invention also encompasses amino acid sequences of polypeptides having the specific properties as defined herein.

25   As used herein, the term "amino acid sequence" is synonymous with the term "polypeptide" and/or the term "protein". In some instances, the term "amino acid sequence" is synonymous with the term "peptide".

The amino acid sequence may be prepared/isolated from a suitable source, or it may be
30   made synthetically or it may be prepared by use of recombinant DNA techniques.

Suitably, the amino acid sequences may be obtained from the isolated polypeptides taught herein by standard techniques.

One suitable method for determining amino acid sequences from isolated polypeptides
5   is as follows:

Purified polypeptide may be freeze-dried and 100 μg of the freeze-dried material may be dissolved in 50 μl of a mixture of 8 M urea and 0.4 M ammonium hydrogen carbonate, pH 8.4. The dissolved protein may be denatured and reduced for 15 minutes
10   at 50°C following overlay with nitrogen and addition of 5 μl of 45 mM dithiothreitol. After cooling to room temperature, 5 μl of 100 mM iodoacetamide may be added for the cysteine residues to be derivatized for 15 minutes at room temperature in the dark under nitrogen.

15   135 μl of water and 5 μg of endoproteinase Lys-C in 5 μl of water may be added to the above reaction mixture and the digestion may be carried out at 37°C under nitrogen for 24 hours.

The resulting peptides may be separated by reverse phase HPLC on a VYDAC C18
20   column (0.46x15cm;10μm; The Separation Group, California, USA) using solvent A: 0.1% TFA in water and solvent B: 0.1% TFA in acetonitrile. Selected peptides may be re-chromatographed on a Develosil C18 column using the same solvent system, prior to N-terminal sequencing. Sequencing may be done using an Applied Biosystems 476A sequencer using pulsed liquid fast cycles according to the manufacturer's
25   instructions (Applied Biosystems, California, USA).

SEQUENCE IDENTITY OR SEQUENCE HOMOLOGY

30   The present invention also encompasses the use of sequences having a degree of sequence identity or sequence homology with amino acid sequence(s) of a polypeptide having the specific properties defined herein or of any nucleotide sequence encoding

such a polypeptide (hereinafter referred to as a "homologous sequence(s)"). Here, the term "homologue" means an entity having a certain homology with the subject amino acid sequences and the subject nucleotide sequences. Here, the term "homology" can be equated with "identity".

5

The homologous amino acid sequence and/or nucleotide sequence should provide and/or encode a polypeptide which retains the functional activity and/or enhances the activity of the enzyme.

10   In the present context, a homologous sequence is taken to include an amino acid sequence which may be at least 75, 85 or 90% identical, preferably at least 95 or 98% identical to the subject sequence. Typically, the homologues will comprise the same active sites etc. as the subject amino acid sequence. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical

15   properties/functions), in the context of the present invention it is preferred to express homology in terms of sequence identity.

In the present context, a homologous sequence is taken to include a nucleotide sequence which may be at least 75, 85 or 90% identical, preferably at least 95 or 98% identical to a nucleotide sequence encoding a polypeptide of the present invention (the subject sequence). Typically, the homologues will comprise the same sequences that code for the active sites etc. as the subject sequence. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express

25   homology in terms of sequence identity.

Homology comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs can calculate % homology between two or more sequences.

30

% homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence is directly

compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues.

5   Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to
10   produce optimal alignments that take into consideration possible insertions and deletions without penalising unduly the overall homology score. This is achieved by inserting "gaps" in the sequence alignment to try to maximise local homology.

However, these more complex methods assign "gap penalties" to each gap that occurs
15   in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. "Affine gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most
20   commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package the default gap penalty for amino acid sequences is -12 for
25   a gap and -4 for each extension.

Calculation of maximum % homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package
30   (Devereux et al 1984 Nuc. Acids Research 12 p387). Examples of other software that can perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel et al 1999 Short Protocols in Molecular Biology, 4th Ed – Chapter 18),

FASTA (Altschul *et al* 1990 J. Mol. Biol. 403-410) and the GENEWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching (see Ausubel *et al* 1999, pages 7-58 to 7-60). However, for some applications, it is preferred to use the GCG Bestfit program. A new tool, called

5 BLAST 2 Sequences is also available for comparing protein and nucleotide sequence (see FEMS Microbiol Lett 1999 174(2): 247-50; FEMS Microbiol Lett 1999 177(1): 187-8 and tatiana@ncbi.nlm.nih.gov).

Although the final % homology can be measured in terms of identity, the alignment

10 process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public

15 default values or a custom symbol comparison table if supplied (see user manual for further details). For some applications, it is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

20 Alternatively, percentage homologies may be calculated using the multiple alignment feature in DNASIS™ (Hitachi Software), based on an algorithm, analogous to CLUSTAL (Higgins DG & Sharp PM (1988), *Gene* 73(1), 237-244).

Once the software has produced an optimal alignment, it is possible to calculate %

25 homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

The sequences may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent

30 substance. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the secondary binding activity of the substance is

retained. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include. leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine,
5   phenylalanine, and tyrosine.

Conservative substitutions may be made, for example according to the Table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:
10

ALIPHATIC	Non-polar	G A P
		I L V
	Polar — uncharged	C S T M
		N Q
	Polar — charged	D E
		K R
AROMATIC		H F W Y

The present invention also encompasses homologous substitution (substitution and replacement are both used herein to mean the interchange of an existing amino acid
15   residue, with an alternative residue) that may occur i.e. like-for-like substitution such as basic for basic, acidic for acidic, polar for polar etc. Non-homologous substitution may also occur i.e. from one class of residue to another or alternatively involving the inclusion of unnatural amino acids such as ornithine (hereinafter referred to as Z), diaminobutyric acid ornithine (hereinafter referred to as B), norleucine ornithine
20   (hereinafter referred to as O), pyriylalanine, thienylalanine, naphthylalanine and phenylglycine.

Replacements may also be made by unnatural amino acids.

Variant amino acid sequences may include suitable spacer groups that may be inserted between any two amino acid residues of the sequence including alkyl groups such as methyl, ethyl or propyl groups in addition to amino acid spacers such as glycine or β-alanine residues. A further form of variation, involves the presence of one or more

5   amino acid residues in peptoid form, will be well understood by those skilled in the art. For the avoidance of doubt, "the peptoid form" is used to refer to variant amino acid residues wherein the α-carbon substituent group is on the residue's nitrogen atom rather than the α-carbon. Processes for preparing peptides in the peptoid form are known in the art, for example Simon RJ et al., PNAS (1992) 89(20), 9367-9371 and

10   Horwell DC, Trends Biotechnol. (1995) 13(4), 132-134.

Nucleotide sequences for use in the present invention or encoding a polypeptide having the specific properties defined herein may include within them synthetic or modified nucleotides. A number of different types of modification to oligonucleotides

15   are known in the art. These include methylphosphonate and phosphorothioate backbones and/or the addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the nucleotide sequences described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity

20   or life span of nucleotide sequences.

The present invention also encompasses the use of nucleotide sequences that are complementary to the sequences discussed herein, or any derivative, fragment or derivative thereof. If the sequence is complementary to a fragment thereof then that

25   sequence can be used as a probe to identify similar coding sequences in other organisms etc.

Polynucleotides which are not 100% homologous to the sequences of the present invention but fall within the scope of the invention can be obtained in a number of ways.

30   Other variants of the sequences described herein may be obtained for example by probing DNA libraries made from a range of individuals, for example individuals from different populations. In addition, other viral/bacterial, or cellular homologues particularly cellular

homologues found in mammalian cells (e.g. rat, mouse, bovine and primate cells), may be obtained and such homologues and fragments thereof in general will be capable of selectively hybridising to the sequences shown in the sequence listing herein. Such sequences may be obtained by probing cDNA libraries made from or genomic DNA

5   libraries from other animal species, and probing such libraries with probes comprising all or part of any one of the sequences in the attached sequence listings under conditions of medium to high stringency. Similar considerations apply to obtaining species homologues and allelic variants of the polypeptide or nucleotide sequences of the invention.

10

Variants and strain/species homologues may also be obtained using degenerate PCR which will use primers designed to target sequences within the variants and homologues encoding conserved amino acid sequences within the sequences of the present invention. Conserved sequences can be predicted, for example, by aligning the amino acid

15   sequences from several variants/homologues. Sequence alignments can be performed using computer software known in the art. For example the GCG Wisconsin PileUp program is widely used.

The primers used in degenerate PCR will contain one or more degenerate positions and

20   will be used at stringency conditions lower than those used for cloning sequences with single sequence primers against known sequences.

Alternatively, such polynucleotides may be obtained by site directed mutagenesis of characterised sequences. This may be useful where for example silent codon sequence

25   changes are required to optimise codon preferences for a particular host cell in which the polynucleotide sequences are being expressed. Other sequence changes may be desired in order to introduce restriction polypeptide recognition sites, or to alter the property or function of the polypeptides encoded by the polynucleotides.

30   Polynucleotides (nucleotide sequences) of the invention may be used to produce a primer, e.g. a PCR primer, a primer for an alternative amplification reaction, a probe e.g. labelled with a revealing label by conventional means using radioactive or non-radioactive labels,

or the polynucleotides may be cloned into vectors. Such primers, probes and other fragments will be at least 15, preferably at least 20, for example at least 25, 30 or 40 nucleotides in length, and are also encompassed by the term polynucleotides of the invention as used herein.

5

Polynucleotides such as DNA polynucleotides and probes according to the invention may be produced recombinantly, synthetically, or by any means available to those of skill in the art. They may also be cloned by standard techniques.

10 In general, primers will be produced by synthetic means, involving a stepwise manufacture of the desired nucleic acid sequence one nucleotide at a time. Techniques for accomplishing this using automated techniques are readily available in the art.

Longer polynucleotides will generally be produced using recombinant means, for
15 example using a PCR (polymerase chain reaction) cloning techniques. This will involve making a pair of primers (e.g. of about 15 to 30 nucleotides) flanking a region of the lipid targeting sequence which it is desired to clone, bringing the primers into contact with mRNA or cDNA obtained from an animal or human cell, performing a polymerase chain reaction under conditions which bring about amplification of the desired region, isolating
20 the amplified fragment (e.g. by purifying the reaction mixture on an agarose gel) and recovering the amplified DNA. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector.

HYBRIDISATION

25

The present invention also encompasses sequences that are complementary to the sequences of the present invention or sequences that are capable of hybridising either to the sequences of the present invention or to sequences that are complementary thereto.

30

The term "hybridisation" as used herein shall include "the process by which a strand of nucleic acid joins with a complementary strand through base pairing" as well as the process of amplification as carried out in polymerase chain reaction (PCR) technologies.

5

The present invention also encompasses the use of nucleotide sequences that are capable of hybridising to the sequences that are complementary to the subject sequences discussed herein, or any derivative, fragment or derivative thereof.

10   The present invention also encompasses sequences that are complementary to sequences that are capable of hybridising to the nucleotide sequences discussed herein.

Hybridisation conditions are based on the melting temperature (Tm) of the nucleotide binding complex, as taught in Berger and Kimmel (1987, Guide to Molecular Cloning

15   Techniques, Methods in Enzymology, Vol. 152, Academic Press, San Diego CA), and confer a defined "stringency" as explained below.

Maximum stringency typically occurs at about Tm-5°C (5°C below the Tm of the probe); high stringency at about 5°C to 10°C below Tm; intermediate stringency at

20   about 10°C to 20°C below Tm; and low stringency at about 20°C to 25°C below Tm. As will be understood by those of skill in the art, a maximum stringency hybridisation can be used to identify or detect identical nucleotide sequences while an intermediate (or low) stringency hybridisation can be used to identify or detect similar or related polynucleotide sequences.

25

Preferably, the present invention encompasses sequences that are complementary to sequences that are capable of hybridising under high stringency conditions or intermediate stringency conditions to nucleotide sequences encoding polypeptides having the specific properties as defined herein.

30

More preferably, the present invention encompasses sequences that are complementary to sequences that are capable of hybridising under high stringent conditions (e.g. 65°C

and 0.1xSSC {1xSSC = 0.15 M NaCl, 0.015 M Na-citrate pH 7.0}) to nucleotide sequences encoding polypeptides having the specific properties as defined herein.

The present invention also relates to nucleotide sequences that can hybridise to the
5   nucleotide sequences discussed herein (including complementary sequences of those discussed herein).

The present invention also relates to nucleotide sequences that are complementary to sequences that can hybridise to the nucleotide sequences discussed herein (including
10   complementary sequences of those discussed herein).

Also included within the scope of the present invention are polynucleotide sequences that are capable of hybridising to the nucleotide sequences discussed herein under conditions of intermediate to maximal stringency.
15

In a preferred aspect, the present invention covers nucleotide sequences that can hybridise to the nucleotide sequences discussed herein, or the complement thereof, under stringent conditions (e.g. 50°C and 0.2xSSC).

20   In a more preferred aspect, the present invention covers nucleotide sequences that can hybridise to the nucleotide sequences discussed herein, or the complement thereof, under high stringent conditions (e.g. 65°C and 0.1xSSC).

EXPRESSION OF POLYPEPTIDES
25

A nucleotide sequence for use in the present invention or for encoding a polypeptide having the specific properties as defined herein can be incorporated into a recombinant replicable vector. The vector may be used to replicate and express the nucleotide sequence, in polypeptide form, in and/or from a compatible host cell. Expression may
30   be controlled using control sequences which include promoters/enhancers and other expression regulation signals. Prokaryotic promoters and promoters functional in eukaryotic cells may be used. Tissue specific or stimuli specific promoters may be

used. Chimeric promoters may also be used comprising sequence elements from two or more different promoters described above.

The polypeptide produced by a host recombinant cell by expression of the nucleotide sequence may be secreted or may be contained intracellularly depending on the sequence and/or the vector used. The coding sequences can be designed with signal sequences which direct secretion of the substance coding sequences through a particular prokaryotic or eukaryotic cell membrane.

EXPRESSION VECTOR

The term "expression vector" means a construct capable of *in vivo* or *in vitro* expression.

Preferably, the expression vector is incorporated in the genome of the organism. The term "incorporated" preferably covers stable incorporation into the genome.

The nucleotide sequence of the present invention or coding for a polypeptide having the specific properties as defined herein may be present in a vector, in which the nucleotide sequence is operably linked to regulatory sequences such that the regulatory sequences are capable of providing the expression of the nucleotide sequence by a suitable host organism, i.e. the vector is an expression vector.

The vectors of the present invention may be transformed into a suitable host cell as described below to provide for expression of a polypeptide having the specific properties as defined herein.

The choice of vector, e.g. plasmid, cosmid, virus or phage vector, will often depend on the host cell into which it is to be introduced.

The vectors may contain one or more selectable marker genes – such as a gene which confers antibiotic resistance e.g. ampicillin, kanamycin, chloramphenicol or tetracyclin

resistance. Alternatively, the selection may be accomplished by co-transformation (as described in WO91/17243).

Vectors may be used *in vitro*, for example for the production of RNA or used to
5 transfect or transform a host cell.

Thus, in a further embodiment, the invention provides a method of making nucleotide sequences of the present invention or nucleotide sequences encoding polypeptides having the specific properties as defined herein by introducing a nucleotide sequence
10 into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about replication of the vector.

The vector may further comprise a nucleotide sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication
15 of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

REGULATORY SEQUENCES

In some applications, a nucleotide sequence for use in the present invention or a
20 nucleotide sequence encoding a polypeptide having the specific properties as defined herein may be operably linked to a regulatory sequence which is capable of providing for the expression of the nucleotide sequence, such as by the chosen host cell. By way of example, the present invention covers a vector comprising the nucleotide sequence of the present invention operably linked to such a regulatory sequence, i.e. the vector is
25 an expression vector.

The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence "operably linked" to a coding sequence is ligated in such a way
30 that expression of the coding sequence is achieved under conditions compatible with the control sequences.

The term "regulatory sequences" includes promoters and enhancers and other expression regulation signals.

The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase
5   binding site.

Enhanced expression of the nucleotide sequence encoding the enzyme having the specific properties as defined herein may also be achieved by the selection of heterologous regulatory regions, e.g. promoter, secretion leader and terminator
10   regions.

Preferably, the nucleotide sequence of the present invention may be operably linked to at least a promoter.

15   Examples of suitable promoters for directing the transcription of the nucleotide
     sequence in a bacterial, fungal or yeast host are well known in the art.

CONSTRUCTS

The term "construct" - which is synonymous with terms such as "conjugate", "cassette"
20   and "hybrid" - includes a nucleotide sequence encoding a polypeptide having the specific properties as defined herein for use according to the present invention directly or indirectly attached to a promoter. An example of an indirect attachment is the provision of a suitable spacer group such as an intron sequence, such as the Sh1-intron or the ADH intron, intermediate the promoter and the nucleotide sequence of the present invention.
25   The same is true for the term "fused" in relation to the present invention which includes direct or indirect attachment. In some cases, the terms do not cover the natural combination of the nucleotide sequence coding for the protein ordinarily associated with the wild type gene promoter and when they are both in their natural environment.

30   The construct may even contain or express a marker which allows for the selection of the genetic construct.

For some applications, preferably the construct comprises at least a nucleotide sequence of the present invention or a nucleotide sequence encoding a polypeptide having the specific properties as defined herein operably linked to a promoter.

5

HOST CELLS

The term "host cell" - in relation to the present invention includes any cell that comprises either a nucleotide sequence encoding a polypeptide having the specific

10   properties as defined herein or an expression vector as described above and which is used in the recombinant production of a polypeptide having the specific properties as defined herein.

Thus, a further embodiment of the present invention provides host cells transformed or

15   transfected with a nucleotide sequence of the present invention or a nucleotide sequence that expresses a polypeptide having the specific properties as defined herein. The cells will be chosen to be compatible with the said vector and may for example be prokaryotic (for example bacterial), fungal, yeast or plant cells. Preferably, the host cells are not human cells.

20

Examples of suitable bacterial host organisms are gram negative bacterium or gram positive bacteria.

Depending on the nature of the nucleotide sequence encoding a polypeptide having the

25   specific properties as defined herein, and/or the desirability for further processing of the expressed protein, eukaryotic hosts such as yeasts or other fungi may be preferred. In general, yeast cells are preferred over fungal cells because they are easier to manipulate. However, some proteins are either poorly secreted from the yeast cell, or in some cases are not processed properly (e.g. hyperglycosylation in yeast). In these

30   instances, a different fungal host organism should be selected.

The use of suitable host cells, such as yeast, fungal and plant host cells – may provide for post-translational modifications (e.g. myristoylation, glycosylation, truncation, lapidation and tyrosine, serine or threonine phosphorylation) as may be needed to confer optimal biological activity on recombinant expression products of the present

5   invention.

The host cell may be a protease deficient or protease minus strain.

ORGANISM

10

The term "organism" in relation to the present invention includes any organism that could comprise a nucleotide sequence according to the present invention or a nucleotide sequence encoding for a polypeptide having the specific properties as defined herein and/or products obtained therefrom.

15

Suitable organisms may include a prokaryote, fungus, yeast or a plant.

The term "transgenic organism" in relation to the present invention includes any organism that comprises a nucleotide sequence coding for a polypeptide having the specific properties as defined herein and/or the products obtained therefrom, and/or

20   wherein a promoter can allow expression of the nucleotide sequence coding for a polypeptide having the specific properties as defined herein within the organism. Preferably the nucleotide sequence is incorporated in the genome of the organism.

25   The term "transgenic organism" does not cover native nucleotide coding sequences in their natural environment when they are under the control of their native promoter which is also in its natural environment.

Therefore, the transgenic organism of the present invention includes an organism

30   comprising any one of, or combinations of, a nucleotide sequence coding for a polypeptide having the specific properties as defined herein, constructs as defined herein, vectors as defined herein, plasmids as defined herein, cells as defined herein, or

the products thereof. For example the transgenic organism can also comprise a nucleotide sequence coding for a polypeptide having the specific properties as defined herein under the control of a heterologous promoter.

5   TRANSFORMATION OF HOST CELLS/ORGANISM

As indicated earlier, the host organism can be a prokaryotic or a eukaryotic organism. Examples of suitable prokaryotic hosts include *E. coli* and *Bacillus subtilis*.

10   Teachings on the transformation of prokaryotic hosts is well documented in the art, for example see Sambrook *et al* (Molecular Cloning: A Laboratory Manual, 2nd edition, 1989, Cold Spring Harbor Laboratory Press). If a prokaryotic host is used then the nucleotide sequence may need to be suitably modified before transformation - such as by removal of introns.

15
. In another embodiment the transgenic organism can be a yeast.

Filamentous fungi cells may be transformed using various methods known in the art – such as a process involving protoplast formation and transformation of the protoplasts

20   followed by regeneration of the cell wall in a manner known. The use of *Aspergillus* as a host microorganism is described in EP 0 238 023.

Another host organism can be a plant. A review of the general techniques used for transforming plants may be found in articles by Potrykus (*Annu Rev Plant Physiol*

25   *Plant Mol Biol* [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27). Further teachings on plant transformation may be found in EP-A-0449375.

General teachings on the transformation of fungi, yeasts and plants are presented in

30   following sections.

TRANSFORMED FUNGUS

A host organism may be a fungus - such as a filamentous fungus. Examples of suitable such hosts include any member belonging to the genera Thermomyces, Acremonium,

5   Aspergillus, Penicillium, Mucor, Neurospora, Trichoderma and the like.

Teachings on transforming filamentous fungi are reviewed in US-A-5741665 which states that standard techniques for transformation of filamentous fungi and culturing the fungi are well known in the art. An extensive review of techniques as applied to *N.*

10   *crassa* is found, for example in Davis and de Serres, *Methods Enzymol* (1971) 17A: 79-143.

Further teachings on transforming filamentous fungi are reviewed in US-A-5674707.

15   In one aspect, the host organism can be of the genus *Aspergillus*, such as *Aspergillus niger*.

A transgenic *Aspergillus* according to the present invention can also be prepared by following, for example, the teachings of Turner G. 1994 (Vectors for genetic

20   manipulation. In: Martinelli S.D., Kinghorn J.R.(Editors) *Aspergillus*: 50 years on. Progress in industrial microbiology vol 29. Elsevier Amsterdam 1994. pp. 641-666).

Gene expression in filamentous fungi has been reviewed in Punt *et al.* (2002) Trends Biotechnol 2002 May;20(5):200-6, Archer & Peberdy Crit Rev Biotechnol (1997)

25   17(4):273-306.

TRANSFORMED YEAST

In another embodiment, the transgenic organism can be a yeast.

30

A review of the principles of heterologous gene expression in yeast are provided in, for example, *Methods Mol Biol* (1995), 49:341-54, and *Curr Opin Biotechnol* (1997) Oct;8(5):554-60

5   In this regard, yeast – such as the species *Saccharomyces cerevisi* or *Pichia pastoris* (see FEMS Microbiol Rev (2000 24(1):45-66), may be used as a vehicle for heterologous gene expression.

A review of the principles of heterologous gene expression in *Saccharomyces cerevisiae*
10   and secretion of gene products is given by E Hinchcliffe E Kenny (1993, "Yeast as a vehicle for the expression of heterologous genes", *Yeasts*, Vol 5, Anthony H Rose and J Stuart Harrison, eds, 2nd edition, Academic Press Ltd.).

For the transformation of yeast, several transformation protocols have been developed.
15   For example, a transgenic Saccharomyces according to the present invention can be prepared by following the teachings of Hinnen *et al.*, (1978, *Proceedings of the National Academy of Sciences of the USA* 75, 1929); Beggs, J D (1978, *Nature*, London, 275, 104); and Ito, H *et al* (1983, J Bacteriology 153, 163-168).

20   The transformed yeast cells may be selected using various selective markers – such as auxotrophic markers dominant antibiotic resistance markers.

TRANSFORMED PLANTS/PLANT CELLS

25   A host organism suitable for the present invention may be a plant. A review of the general techniques may be found in articles by Potrykus (*Annu Rev Plant Physiol Plant Mol Biol* [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27), or in WO01/16308. The transgenic plant may produce enhanced levels of phytosterol esters and phytostanol esters, for example.

30

Therefore the present invention also relates to a method for the production of a transgenic plant with enhanced levels of phytosterol esters and phytostanol esters, comprising the

steps of transforming a plant cell with a lipid acyltransferase as defined herein (in particular with an expression vector or construct comprising a lipid acyltransferase as defined herein), and growing a plant from the transformed plant cell.

5   SECRETION

Often, it is desirable for the polypeptide to be secreted from the expression host into the culture medium from where the enzyme may be more easily recovered. According to the present invention, the secretion leader sequence may be selected on the basis of

10   the desired expression host. Hybrid signal sequences may also be used with the context of the present invention.

Typical examples of heterologous secretion leader sequences are those originating from the fungal amyloglucosidase (AG) gene (glaA - both 18 and 24 amino acid

15   versions e.g. from Aspergillus), the a-factor gene (yeasts e.g. Saccharomyces, Kluyveromyces and Hansenula) or the α-amylase gene (Bacillus).

DETECTION

20   A variety of protocols for detecting and measuring the expression of the amino acid sequence are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS).

25   A wide variety of labels and conjugation techniques are known by those skilled in the art and can be used in various nucleic and amino acid assays.

A number of companies such as Pharmacia Biotech (Piscataway, NJ), Promega (Madison, WI), and US Biochemical Corp (Cleveland, OH) supply commercial kits

30   and protocols for these procedures.

Suitable reporter molecules or labels include those radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles and the like. Patents teaching the use of such labels include US-A-3,817,837; US-A-3,850,752; US-A-3,939,350; US-A-3,996,345; US-A-
5   4,277,437; US-A-4,275,149 and US-A-4,366,241.

Also, recombinant immunoglobulins may be produced as shown in US-A-4,816,567.

10   FUSION PROTEINS

A polypeptide having the specific properties as defined herein may be produced as a fusion protein, for example to aid in extraction and purification thereof. Examples of fusion protein partners include glutathione-S-transferase (GST), 6xHis, GAL4 (DNA
15   binding and/or transcriptional activation domains) and β-galactosidase. It may also be convenient to include a proteolytic cleavage site between the fusion protein partner and the protein sequence of interest to allow removal of fusion protein sequences. Preferably the fusion protein will not hinder the activity of the protein sequence.

20   Gene fusion expression systems in E. coli have been reviewed in Curr. Opin. Biotechnol. (1995) 6(5):501-6.

In another embodiment of the invention, the amino acid sequence of a polypeptide having the specific properties as defined herein may be ligated to a heterologous
25   sequence to encode a fusion protein. For example, for screening of peptide libraries for agents capable of affecting the substance activity, it may be useful to encode a chimeric substance expressing a heterologous epitope that is recognised by a commercially available antibody.

30   The invention will now be described, by way of example only, with reference to the following Figures and Examples.

Figure 1 shows a pfam00657 consensus sequence from database version 6 (SEQ ID No. 1);

Figure 2 shows an amino acid sequence (SEQ ID No. 2) obtained from the organism
Aeromonas hydrophila (P10480; GI:121051);

Figure 3 shows an amino acid sequence (SEQ ID No. 3) obtained from the organism
Aeromonas salmonicida (AAG098404; GI:9964017);

Figure 4 shows an amino acid sequence (SEQ ID No. 4) obtained from the organism
Streptomyces coelicolor A3(2) (Genbank accession number NP_631558);

Figure 5 shows an amino acid sequence (SEQ ID No. 5) obtained from the organism
Streptomyces coelicolor A3(2) (Genbank accession number: CAC42140);

Figure 6 shows an amino acid sequence (SEQ ID No. 6) obtained from the organism
Saccharomyces cerevisiae (Genbank accession number P41734);

Figure 7 shows an alignment of selected sequences to pfam00657 consensus sequence;

Figure 8 shows a pairwise alignment of SEQ ID No. 3 with SEQ ID No. 2 showing
93% amino acid sequence identity. The signal sequence is underlined. + denotes
differences. The GDSX motif containing the active site serine 16, and the active sites
aspartic acid 116 and histidine 291 are highlighted (see shaded regions). Numbers after
the amino acid is minus the signal sequence;

Figure 9 shows a nucleotide sequence (SEQ ID No. 7) encoding a lipid acyl transferase
according to the present invention obtained from the organism *Aeromonas hydrophila;*

Figure 10 shows a nucleotide sequence (SEQ ID No. 8) encoding a lipid acyl
transferase according to the present invention obtained from the organism *Aeromonas
salmonicida;*

Figure 11 shows a nucleotide sequence (SEQ ID No. 9) encoding a lipid acyl transferase according to the present invention obtained from the organism *Streptomyces coelicolor* A3(2) (Genbank accession number

5   NC_003888.1:8327480..8328367);

Figure 12 shows a nucleotide sequence (SEQ ID No. 10) encoding a lipid acyl transferase according to the present invention obtained from the organism *Streptomyces coelicolor* A3(2) (Genbank accession number

10   AL939131.1:265480..266367);

Figure 13 shows a nucleotide sequence (SEQ ID No. 11) encoding a lipid acyl transferase according to the present invention obtained from the organism *Saccharomyces cerevisiae* (Genbank accession number Z75034);

15

Figure 14 shows an amino acid sequence (SEQ ID No. 12) obtained from the organism *Ralstonia* (Genbank accession number: AL646052);

Figure 15 shows a nucleotide sequence (SEQ ID No. 13) encoding a lipid acyl

20   transferase according to the present invention obtained from the organism *Ralstonia*;

Figure 16 shows SEQ ID No. 20. Scoe1 NCBI protein accession code CAB39707.1 GI:4539178 conserved hypothetical protein [Streptomyces coelicolor A3(2)];

25   Figure 17 shows a nucleotide sequence shown as SEQ ID No. 21 encoding NCBI protein accession code CAB39707.1 GI:4539178 conserved hypothetical protein [Streptomyces coelicolor A3(2)];

Figure 18 shows an amino acid shown as SEQ ID No.22. Scoe2 NCBI protein

30   accession code CAC01477.1 GI:9716139 conserved hypothetical protein [Streptomyces coelicolor A3(2)];

Figure 19 shows a nucleotide sequence shown as SEQ ID No. 23 encoding Scoe2 NCBI protein accession code CAC01477.1 GI:9716139 conserved hypothetical protein [Streptomyces coelicolor A3(2)];

5   Figure 20 shows an amino acid sequence (SEQ ID No.24) Scoe3 NCBI protein accession code CAB88833.1 GI:7635996 putative secreted protein. [Streptomyces coelicolor A3(2)];

Figure 21 shows a nucleotide sequence shown as SEQ ID No. 25 encoding Scoe3

10   NCBI protein accession code CAB88833.1 GI:7635996 putative secreted protein. [Streptomyces coelicolor A3(2)];

Figure 22 shows an amino acid sequence (SEQ ID No.26) Scoe4 NCBI protein accession code CAB89450.1 GI:7672261 putative secreted protein. [Streptomyces

15   coelicolor A3(2)];

Figure 23 shows an nucleotide sequence shown as SEQ ID No. 27 encoding Scoe4 NCBI protein accession code CAB89450.1 GI:7672261 putative secreted protein. [Streptomyces coelicolor A3(2)];

20

Figure 24 shows an amino acid sequence (SEQ ID No.28) Scoe5 NCBI protein accession code CAB62724.1 GI:6562793 putative lipoprotein [Streptomyces coelicolor A3(2)];

25   Figure 25 shows a nucleotide sequence shown as SEQ ID No. 29, encoding Scoe5 NCBI protein accession code CAB62724.1 GI:6562793 putative lipoprotein [Streptomyces coelicolor A3(2)];

Figure 26 shows an amino acid sequence (SEQ ID No.30) Sriml NCBI protein

30   accession code AAK84028.1 GI:15082088 GDSL-lipase [Streptomyces rimosus];

Figure 27 shows a nucleotide sequence shown as SEQ ID No. 31 encoding Srim1 NCBI protein accession code AAK84028.1 GI:15082088 GDSL-lipase [Streptomyces rimosus];

5   Figure 28 shows an amino acid sequence (SEQ ID No.32)A lipid acyl transferase from *Aeromonas hydrophila* (ATCC #7965);

Figure 29 shows a nucleotide sequence (SEQ ID No. 33) encoding a lipid acyltransferase from *Aeromonas hydrophila* (ATCC #7965);

10

Figure 30 shows an amino acid sequence (SEQ ID No.34) of a lipid acyltransferase from *Aeromonas salmonicida* subsp. *Salmonicida* (ATCC#14174);

Figure 31 shows a nucleotide sequence (SEQ ID No 35) encoding a lipid
15   acyltransferase from *Aeromonas salmonicida* subsp. *Salmonicida* (ATCC#14174);

Figure 32 shows that homologues of the *Aeromonas* genes can be identified using the basic local alignment search tool service at the National Center for Biotechnology Information, NIH, MD, USA and the completed genome databases. The GDSX motif was used in the database search and a number of sequences/genes potentially encoding
20   enzymes with lipolytic activity were identified. Genes were identified from the genus Streptomyces, Xanthomonas and Ralstonia. As an example below, the Ralstonia solanacearum was aligned to the Aeromonas salmonicida (satA) gene. Pairwise alignment showed 23% identity. The active site serine is present at the amino terminus and the catalytic residues histidine and aspartic acid can be identified;

25

Figure 33 shows the Pfam00657.11 [family 00657, database version 11] consensus sequence (hereafter called Pfam consensus) and the alignment of various sequences to the Pfam consensus sequence. The arrows indicate the active site residues, the underlined boxes indicate three of the homology boxes indicated by [Upton C and
30   Buckley JT (1995) Trends Biochem Sci 20; 179-179]. Capital letters in the Pfam consensus indicate conserved residues in many family members. The – symbol indicates a position where the hidden Markov model of the Pfam consensus expected

to find a residue but did not, so a gap is inserted. The . symbol indicates a residue without a corresponding residue in the Pfam consensus. The sequences are the amino acid sequences listed in Figures 16, 18, 20, 22, 24, 26, 28 and 30.

5 Figure 34 shows the Pfam00657.11 [family 00657, database version 11] consensus sequence (hereafter called Pfam consensus) and the alignment of various sequences to the Pfam consensus sequence. The arrows indicate the active site residues, the underlined boxes indicate three of the homology boxes indicated by [Upton C and Buckley JT (1995) Trends Biochem Sci 20; 179-179]. Capital letters in the Pfam

10 consensus indicate conserved residues in many family members. The − symbol indicates a position where the hidden Markov model of the Pfam consensus expected to find a residue but did not, so a gap is inserted. The . symbol indicates a residue without a corresponding residue in the Pfam consensus. The sequences are the amino acid sequences listed in Figures 2, 16, 18, 20, 26, 28 and 30. All these proteins were

15 found to be active against lipid substrates.

Figure 35 shows a expression vector pet12-AsalGCAT= pSM containing the C-terminal His-tagged *Aeromonas salmonicida* lipid acyltransferase gene;

Figure 36 shows the results of testing cell extracts in a NEFA Kit Assay, which depicts

20 the activity of a recombinant, *A. salmonicida* lipid acyltransferase, towards lecithin. The wells from left to right indicate: a positive control, a negative control (i.e. extracts from empty plasmid) and samples collected after 0, 1, 2 and 3 hours cultivation after IPTG induction;

25 Figure 37 shows growth optimisation of BL21(DE3)pLysS harboring the expression vector pet12-AsalGCAT= pSM showing cultivation at 30 ºC resulted in the production of enzyme with high activity towards lecithin. Cell extracts were tested for phospholipase activity using the NEFA kit assay. Wells from left to right: positive control; negative control; 20ºC; 30ºC;

30

Figure 38 shows crude cell extracts from BL21(DE3)pLysS expressing active lipid acyltransferase incubated with the substrate lecithin and reaction mixture was analyzed

using thin layer chromatography showing the presence of degradation products. Lanes:
1. No enzyme; 2. + A.sal −10ul 37°C; 3. + A. sal −20ul 37°C; 4. + A.sal −
10ul 24°C; 5. + A. sal −20u 24°C;

5 Figure 39 shows partial purification of the *Aeromonas salmonicida* Acyl Transferase.
showing the phospholipase activity associated with purified His-tag protein. SE =
Sonicated extracts, His = Purified with Ni-NTA spin-kit from Qiagen;

Figure 40 shows the expression vector pet12-A.h. GCAT=pSMa containing the C-
10 terminal His-tagged *Aeromonas hydrophila* Glycerolipid Acyl Transferase (GCAT)
gene was used to transform *E.coli* strain BL21(DE3)pLysS;

Figure 41 shows the activity of the crude extracts (5 & 10ul) containing the
recombinant *Aeromonas hydrophila* GCAT enzyme was tested towards lecithin using
15 Non-Esterified Fatty Acid (NEFA) kit (Roche, Switzerland), showing the presence of
active enzyme towards the phospholipid, lecithin;

Figure 42 shows growth optimisation of BL21(DE3)pLysS harboring the expression
vector pet12-AsalGCAT= pSM showing cultivation at 30 °C resulted in the production
20 of enzyme with high activity towards lecithin. Cell extracts were tested for
phospholipase activity using the NEFA kit assay;

Figure 43 shows the partial purification of the *Aeromonas hydrophila* & *A.
salmonicida* Acyl Transferases showing the phospholipase activity associated with
25 purified His-tag protein. SE = Sonicated extracts,
His = Purified with Ni-NTA spin-kit from Qiagen);

Figure 44 shows the expression of the *Aeromonas* genes in *Bacillus subtilis* 163
showing the production of secreted enzyme with activity towards both lecithin and
30 DGDG. pUB-AH= construct containing the *A. hydrophila* gene and pUB-AS,
construct with the *A. salmonicida* gene, Culture filtrate was incubated with the
substrates for 60 minutes.

Figure 45 and Fig 46 show a TLC plate in developing solvent IV (chloroform:methanol:water (65:25:4)); Lane 1: 40 mg sitosterol 30 min: Lane 2: Transferase+ 40 mg sitosterol 30 min; Lane 3: Transferase+ 80 mg sitosterol 30 min; Lane 4: Transferase+ 40 mg sitosterol 120 min; Lane 5: Transferase+ 80 mg sitosterol 120 min; Lane 6: Transferase+ 40 mg sitosterol 300 min; Lane 7: 40 mg sitosterol 300 min; Lane 8: Cholesterol; Lane 9: Sitosterol;

Figure 47 depicts the reaction between phosphatidylcholine and cholesterol which is catalysed by a lipid acyltransferase;

Figure 48 shows a TLC analysis of lipids extracted from enzyme treated or untreated egg yolk., 6) 0.31PLU/g Transferase #179, 7) 1.25PLU/g Transferase #178-9., 8) 23.25 PLU/g Phospholipase #3108., 9) Control.

Figure 49 shows mayonnaise test samples produced by enzyme treated or untreated egg yolk: 5) Transferase #179, 0.31 PLU/g. 6) Transferase #178-9, 1.25 PLU/g, 7) Phospholipase #3108, 23.3 PLU/g 8) Control, water

Figure 50 shows a TLC (in solvent I) of egg yolk lipid treated with a lipid acyl transferase from *A. hydrophila*;

Figure 51 shows a TLC (in solvent IV) of egg yolk lipid treated with a lipid acyl transferase from *A. hydrophila*;

Figure 52 shows a TLC analysis of transferase treated lipid from egg yolk over a time course;

Figure 53 shows the amount of fatty acid and cholesterol ester produced as a function of time when using a lipid acyltransferase (Tranf #178-9) compared with when using a control lipolytic enzyme, *Thermomyces lanuginosus*;

Figure 54 shows relative transferase activity as % of transferase and hydrolytic activity in enzymatic reactions in egg yolk with high water content, #1991 and #2427 are control phospholipase A2s, #178 is a lipid acyltransferase;

5 Figure 55 shows the effect of water content in the assay on the transferase activity of the transferase #178 in transferase reactions in egg yolk with high water content.

Figure 56 shows the transferase activity for a lipid acyltransferase (#178) as a function of reaction time in transferase reactions in egg yolk with high water.

10

Figure 57 and Figure 58 show graphs depicting fatty acid and cholesterol ester as a function of time. The graphs depict results obtained for GLC analysis in the assay for measurement of acyltransferase activity in a foodstuff using lecithin and cholesterol in buffer as substrate

15

Figure 59 shows a TLC in solvent I. Egg yolk treated with lipid acyltransferase #138 from *Aeromonas salmonidica* (lane no. 1 and 2) or with a phospholipase #2938 (LIPOPAN® F) (lane no. 3) or Untreated egg yolk (lane no. 4);

20 Figure 60 shows a TLC in solvent IV. Egg yolk treated with lipid acyltransferase #138 (lane no. 1 and 2) and with Phospholipase #2938 (lane no. 3). Untreated egg yolk (lane no. 4);

Figure 61 shows egg yolk treated with lipid acyltransferase #138 (sample nos. 1 and 2) 25 and with phospholipase #2938 (sample no. 3). Untreated egg yolk (sample no. 4);

Figure 62 shows a mayonnaise emulsion after 2 hours at 100 °C. 0) Untreated egg yolk 1)Egg yolk treated with lipid acyl transferase #138 for 210 minutes. 3) Egg yolk treated with the control phospholipase #2938 for 210 minutes;

30

Figure 63 shows GLC plates showing the screening of transferase activity on plant sterol and glycerol. PC = Phosphatidyl cholin, LPC = Lysophosphatidyl cholin; PE = plant esterol; monogl = monoglyceride;

5   Figure 64 shows a TLC plate in solvent I, Samples 1 to 6 after 24 hours and samples 1 to 4 after 4 hours reaction time. The TLC analysis confirms the formation of sterol ester in samples 1, 2, 5 and 6;

Figure 65 shows a TLC plate in solvent I where the transferase activity of an
10   immobilised acyltransferase from *Aeromonas salmonicida* was tested in an oil mixture – with samples taken at 0.5, 1, 3, 6 and 24 h;

Figures 66 and 67 show TLC plates in solvent IV. Lane 1 = lecithin; Lane 2 = control – 10mins; Lane 3 = 0.75 PLU, 10mins; Lane 4 = 0.75 PLU, 60mins; Lane 5 = 0.75
15   PLU, 220mins; Lane 6 = control, 20 h; Lane 7 = 0.75 PLU, 20h; and Lane 8 = cholesterol ester;

Figures 68 and 69 show TLC plates in solvent IV. Lane 1 = lecithin; Lane 2 = control – 10mins; Lane 3 = 1 PLU, 10mins; Lane 4 = 1 PLU, 60mins; Lane 5 = 1 PLU,
20   180mins; Lane 6 = 1PLU, 220mins; Lane 7 = 1PLU, 1200min; Lane 8 = control, 1200 min; Lane 9 = glucose ester; Lane 10 = cholesterol; and Lane 11 = glucose;

Figure 70 shows the reaction between DGDG and sucrose when catalysed by a lipid acyltransferase;
25

Figure 71 shows an amino acid sequence (SEQ ID No. 36) of the fusion construct used for mutagenesis of the *Aeromonas hydrophila* lipid acyltransferase gene in Example 17. The underlined amino acids is a xylanase signal peptide;

30   Figure 72 shows a nucleotide sequence (SEQ ID No. 45) encoding an enzyme from *Aeromonas hydrophila* including a xylanase signal peptide; and

Figure 73 shows a TLC plate clearly showing the formation of plant sterol ester and monoglyceride. Lane 1 is after 1 hour reaction time, Lane 2 is after 4 hours reaction time, Lane 3 is after 24 hours reaction time and Lane 4 is a plant sterol.

5   EXAMPLES

EXAMPLE 1: The cloning, sequencing and heterologous expression of a transferase from *Aeromonas salmonicida* subsp. *Salmonicida*

10

Strains used:

Aeromonas salmonicida subsp. *Salmonicida* (ATCC 14174) was obtained from ATCC and grown overnight at 30°C in Luria-Bertani medium (LB). The cells were centrifuged and genomic DNA was isolated using the procedures for genomic DNA

15   isolation from Qiagen Ltd. Genomic DNA buffer set (cat.19060), protease K (cat. 19131) and RNAse A (cat. 19101) were all obtained from Qiagen Ltd. (Boundary court Gatwick Court, West Sussex, RH10 2AX).

Host bacterial strain BL21(DE3)pLysS (Novagen) was used for production of the

20   recombinant *Aeromonas* enzymes. Competent cells of BL21(DE3)pLysS were used as host for transformation with the expression vector pet12-AsalGCAT=pSM. Transformants containing the appropriate plasmid were grown at 37 °C in LB agar medium containing 100-ug ampicillin/ml.

25

Construction of expression vector pet12-AsalGCAT- pSM:

For all DNA amplifications of the transferase genes from *Aeromonas*, genomic DNA (0.2-1 ul) was used as template and *pfu* DNA polymerase (2.5 units) was used with

30   10ul of 10x pfu buffer, 1ul each primer (50pmol/ul), 200 uMdNTP in a total reaction volume of 100uL. PCR reactions were performed in a programmable thermal cycler using the following conditions: 95 °C for 30 seconds, 30 cycles of 95 °C for 30

seconds, 60 °C for 1 minute and 68 °C for 2 minutes. An additional extension of 5 minutes at 72 °C was applied.

The PCR amplification of the transferase gene from *A. salmonicida* was carried in 2
5   separate PCR reactions. PCR reaction 1 was performed using primer pairs, as1USNEW(5'AGCATATGAAAA AATGGTTTGT TTGTTTATTG GGG 3' [SEQ ID No. 36]) and asls950new (5' GTG ATG GTG GGC GAG GAA CTC GTA CTG3' [SEQ ID No. 37]). A second PCR reaction was performed to incorporate a C-terminal Histidine tag using the PCR product from the first reaction and the primers:
10   as1USNEW(5'AGCATATGAAAA AATGGTTTGT TTGTTTATTG GGG 3' [SEQ ID No. 38]) and AHLS1001(5'TTGGATCC GAATTCAT CAATG GTG ATG GTG ATG GTG GGC3' [SEQ ID No. 39]). The PCR product from the second reaction was purified and digested with restriction enzymes NdeI and BamHI. 2 ug of pET 12a vector DNA was also digested with restriction enzymes NdeI and BamHI and treated
15   with phosphatase. The restriction enzyme-treated pet12a and PCR product from reaction 2 were purified and ligated using the Rapid Ligation Kit (Roche, Switzerland). The ligation mix was used to transform *E. coli* TOP10 cells. Transformants were plated on LB agar medium containing 100ug/ml ampicillin.

20   The T7 promoter primer (5'TAATACGACTCACTATAG3' [SEQ ID No. 40]) and the T7 terminator primer (5'CTAGTTATTGCTCAGCGG3' [SEQ ID No. 41]) were used to verify the sequences and the orientation of the cloned transferase genes in pET12a vector. DNA sequencing was performed using ABI Prism® BigDye™ Terminators Cycle sequencing kit with 500ng plasmid DNA as template and 3.2pmol T7 promoter
25   and terminator primers.

The construct shown in Figure 35 was used to transform competent bacterial host strain BL21(DE3)pLysS (Novagen) and ampicillin resistant transformants were picked and used for expression analysis.

30

Expression of the recombinant *Aeromonas salmonicida* lipid acyltransferase

Quantification of enzyme activity towards lecithin was determined on cell extracts using Non-Esterified Fatty Acid (NEFA) kit (Roche, Switzerland).

5 In Figure 36, BL21(DE3)pLysS harboring the expression vector pet12-AsalGCAT= pSM was grown in LB medium + 100ug/ml ampicillin and incubated with shaking at 37°C until OD_{600} = 0.6 to1.0 is reached. The cultures are then induced using IPTG (0.4mM) and incubation was continued for the next 3 hours. Samples where taken at 0 hour, 1, 2, and 3 hours after IPTG induction. Enzyme Activity was tested using the NEFA kit and lecithin as substrate.

10 Growth Optimisation for the production of more active enzymes

BL21(DE3)pLysS harboring the expression vector pet12-AsalGCAT= pSM was grown in LB medium + 100ug/ml ampicillin and incubated with shaking at different growth temperatures (37°C, 30 °C, & 20 °C). The optimal condition for the production of active lipid acyltransferase enzyme was when cultures are grown at 30°C as shown 15 in Figure 37.

Partial purification of recombinant Aeromonas salmonicida transferase

Strain BL21(DE3)pLysS harboring the expression vector pet12-AsalGCAT=pSM

was grown at 37°C & crude cell extracts were prepared by sonication. The 20 recombinant enzyme was further purified from the sonicated crude cell extracts using the Ni-NTA spin kit from Qiagen. Phospholipase activity using the NEFA kit & Lecithin as substrate. Crude cell extracts from BL21(DE3)pLysS expressing active transferase incubated with the substrate lecithin and reaction mixture was analysed using thin layer chromatography showing the presence of degradation products (see 25 Figure 38).

Partial Purification of recombinant Aeromonas salmonicidae transferase. Strain BL21(DE3)pLysS harbouring the expression vector pet12-AsalGCAT=pSM was grown at 37°C and crude cell extracts were prepared by sonication. The recombinant 30 enzyme ware further purified from the sonicated crude cell extract using the Ni-NTA

spin kit from Qiagen. Phospholipase activity using the NEFA kit and lecithin as substrate was tested (see Figure 39).

5   **EXAMPLE 2 Cloning and Expression of *Aeromonas hydrophila* transferase in *E. coli***

Aeromonas hydrophila (ATCC # 7965) was obtained from ATCC and grown overnight at 30°C in Luria-Bertani medium (LB). The cells were centrifuged and

10   genomic DNA was isolated using the procedures for genomic DNA isolation from Qiagen Ltd. Genomic DNA buffer set (cat.19060), protease K (cat. 19131) and RNAse A (cat. 19101) were all obtained from Qiagen Ltd. (Boundary court Gatwick Court, West Sussex, RH10 2AX).

15   Host bacterial strain BL21(DE3)pLysS (Novagen) was used for production of the recombinant *Aeromonas* enzymes. Competent cells of BL21(DE3)pLysS were used as host for transformation with the expression vector pet12a-A.h.GCAT=pSMa. Transformants containing the appropriate plasmid were grown at 37 °C in LB agar medium containing 100-ug ampicillin/ml.

20

Construction of expression vector pet12a-A.h.GCAT- pSMa:

For all DNA amplifications of the transferase gene from *Aeromonas*, genomic DNA (0.2-1 ul) was used as template and *pfu* DNA polymerase (2.5 units) was used with 10ul of 10x pfu buffer, 1ul each primer (50pmol/ul), 200 uMdNTP in a total reaction

25   volume of 100ul. PCR reactions were performed in a programmable thermal cycler using the following conditions: 95 °C for 30 seconds, 30 cycles of 95 °C for 30 seconds, 60 °C for 1 minute and 68 °C for 2 minutes. An additional extension of 5 minutes at 72 °C was applied.

30   The PCR amplification of the transferase gene from *A. hydrophila* (ATCC # 7965) was carried out in 2 separate PCR reactions.

PCR reaction 1 was performed using primer pairs, AHUS1 (5'GTCATATGAAAAAATGGTTTGTGTGTTTATTGGGATTGGTC3', SEQ ID No. 42) and ahls950 (5'ATGGTGATGGTGGGCGAGGAACTCGTACTG3', SEQ ID No. 43).

A second PCR reaction was performed to incorporate a C-terminal Histidine tag using the PCR product from the first reaction and the primer pairs:

AHUS1(5'GTCATATGAAAAAATGGTTTGTGTGTTTATTGGGATTGGTC3' SEQ ID No. 44,) and AHLS1001(5'TTGGATCCGAATTCATCAATGGTGATGGTGATGGTGGGC3' SEQ ID No. 45).

The PCR product from the second reaction was purified and digested with restriction enzymes Nde1 and BamHI. 2 ug of pET 12a vector DNA was also digested with restriction enzymes Nde1 and BamHI and treated with phosphatase. The restriction enzyme-treated pet12a and PCR product from reaction 2 were purified and ligated using the Rapid Ligation Kit (Roche, Switzerland). The ligation mix was used to transform E. coli TOP10 cells. Transformants were plated on LB agar medium containing 100ug/ml ampicillin.

The T7 promoter primer (5'TAATACGACTCACTATAG3') and the T7 terminator primer (5'CTAGTTATTGCTCAGCGG3') were used to verify the sequences and the orientation of the cloned GCAT genes in pET12a vector. DNA sequencing was performed using ABI Prism® BigDye™ Terminators Cycle sequencing kit with 500ng plasmid DNA as template and 3.2pmol T7 promoter and terminator primers.

The construct shown in Figure 40 was used to transform competent bacterial host strain BL21 (DE3)pLysS (Novagen) and ampicillin resistant transformants were picked and used for expression analysis.

Expression of the *Aeromonas hydrophila* transferase in BL21(DE3)pLysS

The *E. coli* strain BL21(DE3)pLysS harboring the expression vector pet12a-A.h.GCAT= pSMa was grown in LB medium + 100ug/ml ampicillin and incubated with shaking at 37°C until OD_{600} = 0.6 to1.0 is reached. The cultures are then induced
5   using IPTG (0.4mM) and incubation was continued for the next 3 hours. Samples where taken at 0hour, 1, 2, and 3 hours after IPTG induction. Enzyme Activity was tested using the NEFA kit and lecithin as substrate (Figure 41).

Growth Optimisation for the production of more active enzymes

10   BL21(DE3)pLysS harboring the expression vector pet12a-A.h.GCAT= pSMa was grown in LB medium + 100ug/ml ampicillin and incubated with shaking at different growth temperatures (37°C, 30 °C, & 20 °C). The optimal condition for the production of active GCAT enzyme was when cultures are grown at 30°C as shown in Figure 42.

Partial purification of recombinant *A.hydrophila* transferase (GCAT)

15   Strain BL21(DE3)pLysS harboring the expression vector pet12a-A.h.GCAT=pSMa was grown at 37°C & crude cell extracts were prepared by sonication. The recombinant enzyme was further purified from the sonicated crude cell extracts using the Ni-NTA spin kit from Qiagen. Phospholipase activity assay using the NEFA kit & Lecithin as substrate. (Figure 43).

20

EXAMPLE 3: Expression of *Aeromonas* transferases in *Bacillus subtilis* 163

Plasmid Construction

Two different *Bacillus subtilis* expression vectors (pUB 110 & pBE5) were used for the heterologous expression of the *Aeromonas* genes in *Bacillus subtilis*. The pUB110
25   vector contains the alpha amylase promoter while the pBE vector has the P32 promoter as the regulatory region for the expression of the fused *Aeromonas* genes. In pUB110, the first amino acid of the mature GCAT genes of *Aeromonas* were fused in frame with the last amino acid of the xylanase signal peptide sequence from *Bacillus subtilis* via the restriction site Nhe1, creating an additional 2 amino acids in front of the mature

proteins. pBE5 contains the cgtase signal sequence fusion at the NcoI site for secretion of the recombinant proteins into the culture filtrate.

PCR reactions were carried out to obtain the *Aeromonas* genes fuse in frame to the signal sequences of the pUB 110 and the pBE5 vectors. PCRs were performed using the following primer pairs for *A. hydrophila* gene:

PCR reaction 1: usAHncol (5'ATGCCATGGCCGACAGCCGTCCCGCC3', SEQ ID No. 46) and lsAH (5'TTGGATCCGAATTCATCAATGGTGATG3', SEQ ID No. 47)

PCR reaction 2: US-Ahnhel (5'TTGCTAGCGCCGACAGCCGTCCCGCC3', SEQ ID No. 48.) and lsAH (5'TTGGATCCGAATTCATCAATGGTGATG3, SEQ ID No. 49)

PCRs were performed using the following primer pairs for *A. salmonicida* gene:

PCR reaction 3: US-Asncol (5'TTGCCATGGCCGACACTCGCCCCGCC3', SEQ ID No. 50) and lsAH (5'TTGGATCCGAATTCATCAATGGTGATG3', SEQ ID No. 51)

PCR reaction 4: US-ASnhel (5'TTGCTAGCGCCGACACTCGCCCCGCC3', SEQ ID No. 52) and lsAH (5'TTGGATCCGAATTCATCAATGGTGATG3', SEQ ID No. 53)

All the PCR products were cloned into PCR blunt II (TOPO vector) and sequenced with reverse & forward sequencing primers.

Clones from PCR reactions 1 & 3 were cut with NcoI & Bam HI and used as inserts for ligation to the pBE5 vector cut with NcoI/BamHI/phosphatase. Clones from PCR reactions 2 & 4 were cut with NheI & Bam HI and used as inserts for ligation to the pUB vector that was cut with NheI/BamHI/phosphatase.

Expression of the *Aeromonas* transferase genes in *Bacillus subtilis* and characterization of the enzyme activity.

The acyl transferases from the two *Aeromonas* species have been successfully

5   expressed in *E. coli* (results above). The *Bacillus* pUB110 & pBE5 gene fusion constructs were used to transform *Bacillus subtilis* and transformants were selected by plating on kanamycin plates. The kanamycin resistant transformants isolated and grown in 2xYT are capable of heterologous expression of the *Aeromonas* genes in *Bacillus*. The culture filtrates have digalactosyldiacylglycerol (DGDG) galactolipase

10   activity, in addition to having both acyl transferase and phospholipase activities. The activity towards digalactosyldiacylglycerol (DGDG) was measured after 60 minutes of incubation of culture supernatant with the substrate, DGDG from wheat flour (obtainable form Sigma) as well as the activity towards lecithin as shown in Figure 44. *Bacillus* produced the enzyme after overnight (20-24 hours) to 48 hours of cultivation

15   in the culture medium as a secreted protein. In some instances, the expression of the *Aeromonas* genes has been shown to interfere with cell viability and growth in *Bacillus* & *E. coli*, it is therefore necessary to carefully select expression strains and optimise the growth conditions to ensure expression. For example, several *Bacillus* host strains (B.s 163, DB104 and OS 21) were transformed with the expression vectors

20   for growth comparison. B.s163 is transformable with the 2 *Aeromonas* genes and is capable of expressing active protein. DB104 is transformable with all the constructs but is only able to express *A. salmonicida* transferase.

25   **EXAMPLE 4:   Fermentation and Purification of *Aeromonas* lipid acyltransferases produced in *E.coli***

E.coli Fermentations:

Microorganisms

Two strains of *Eschericia coli*, one containing an *Aeromonas hydrophila* (Example 2)

30   lipid acyltransferase and two containing *Aeromonas salmonicida* lipid acyltransferases, (Example 1) were used in this study.

The *E. coli* strain containing the *A. hydrophila* gene was named DIDK0124 , and the *E. coli* strain containing the *A. salmonicida* gene was named DIDK0125. The fermentation with DIDK0124 was named HYDRO0303 and the fermentation with DIDK0125 was named SAL0302. The purified protein from HYDRO025 was named REF#138. The purified protein from HYDRO0303 was named REF#135.

Growth media and culture conditions

LB-agar

The LB agar plates used for maintaining the strains contained: 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 15 g/L agar, 100 mg/L ampicillin and 35 mg/L chloramphenicol. The agar plates were incubated at 30°C.

LB shake flask

The LB medium (50 mL pr shake flask) used for production of inoculum material for the bioreactor cultivations contained: 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 100 mg/L ampicillin and 35 mg/L chloramphenicol. The shake flasks were inoculated from the LB agar plates, and incubated at 30°C and 200 rpm.

Bioreactor cultivation

The bioreactor cultivations were carried out in 6 L in-house built bioreactors filled with 4 L medium containing: 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 8 g/L KH_2PO_4, 0.9 g/L $MgSO_4·7H_2O$, 40 g/L glucose monohydrate, 0.4 mL/ ADD APT® Foamstop Sin 260 (ADD APT Chemicals AG, Helmond, The Netherlands), 10 mg/L $(NH_4)_2Fe(SO_4)_2·6H_2O$, 0.7 mg/L $CuSO_4·5H_2O$, 3 mg/L $ZnSO_4·7H_2O$, 3 mg/L $MnSO_4·H_2O$, 10 mg/L EDTA, 0.1 mg/L $NiSO_4·6H_2O$, 0.1 mg/L $CoCl_2$, 0.1 mg/L H_3BO_4, 0.1 mg/L KI, 0.1 mg/L $Na_2MoO_4·2H_2O$; 1 g/L ampicillin and 35 mg/L chloramphenicol.

The bioreactors were inoculated with an amount of LB culture ensuring end of growth after approximately 20 hours of cultivation (calculated from the maximum specific growth rate of 0.6 h^{-1}, the OD_{600} of the LB shake flask and the final OD_{600} in the bioreactor of approximately 20).

5

SAL0302 was inoculated with 10 mL of LB culture, and HYDRO0303 was inoculated with 4 mL of LB culture.

The bioreactors were operated at the following conditions: temperature 30°C, stirring
10   800-1000 rpm (depending on experiment), aeration 5 L/min, pH 6.9, pH control 8.75% (w/v) NH_3-water and 2 M H_2SO_4. Induction was achieved by addition of isopropyl β-D-thiogalactoside to a final concentration of 0.6 mM, when 0.4 moles (HYDRO0303) and 0.7 moles CO_2 was produced respectively.

15   **Harvest**

The following procedure was used for harvest and homogenisation of the biomass:

1) The fermentation broth from the fermentations was centrifuged at 5000 × g and 4°C for 10 minutes, and the supernatant was discharged. The biomass was stored at –20°C until use. The biomass was thawed and resuspended in 500 mL
20   of 20 mM NaH_2PO_4, pH 7.4, 500 mM NaCl, 10 mM Imidazole and Complete (EDTA-free) protease inhibitor (Roche, Germany).

2) The suspended biomass was homogenized at 2 kbar and 4°C in a cell disrupter from Constant Systems Ltd (Warwick, UK).

3) The cell debris was removed by centrifugation at 10.000 × g and 4°C for 30
25   minutes followed by collection of the supernatant.

4) The supernatant was clarified further by centrifugation at 13.700× g and 4°C for 60 minutes, followed by collection of the supernatant.

5) The supernatant was filtered through 0.2 μm Vacu Cap filters (Pall Life Sciences, UK) and the filtrate was collected for immediate chromatographic
30   purification.

Chromatographic purification of the Transferases

A column (2.5 x 10 cm) was packed with 50 ml of Chelating Sepharose ff. gel and charged with Ni-sulphate (according to the method described by manufacturer, Amersham Biosciences). The column was equilibrated with 200 ml of 20 mM

5   NaH_2PO_4, pH 7.4, 500 mM NaCl, 10 mM Imidazole. 400 ml of crude was applied to the column at a flow rate of 5 ml/min. The column was then washed with 20 mM NaH_2PO_4, pH 7.4, 500 mM NaCl, 10 mM Imidazole until the UV_{280} reached the base line. The GCAT was then eluted with 40 ml of 20 mM NaH_2PO_4, pH 7.4, 500 mM NaCl and 500 mM Imidazole.

10

EXAMPLE 5:   Fermentation and Purification of _Aeromonas_ lipid acyltransferases produced in _Bacillus subtilis_.

Fermentations

15   BAC0318-19, BAC0323-24

Microorganism

The microorganisms used in this study originate from transformation of a _Bacillus subtilis_ host strain, #163 with a plasmid containing the gene encoding the _Aeromonas salmonicida_ transferase inserted in the vector pUB110OIS. The expression of the gene

20   is controlled by an alpha-amylase promoter, and the secretion of the transferase is mediated by the _B. subtilis_ xylanase signal sequence (Example 3). The strains were named DIDK0138 (fermentation BAC0318-19) and DIDK0153 (fermentation BAC0323-24).

25   **Growth media and culture conditions**

Pre culture medium

A shake flask (500 mL total volume, with baffles) was added 100 mL of a medium containing:

NaCl	5 g/L
K_2HPO_4	10 g/L
Soy flour	20 g/L
Yeast extract, BioSpringer 106	20 g/L

30

Antifoam, SIN260   5 mL/L

pH was adjusted to 7.0 before autoclaving

5   After autoclaving 6 mL 50% (w/w) Nutriose were added pr flask. Kanamycin was
     added at a concentration of 50 mg/L after autoclaving.

Inoculation

A pre culture shake flask was inoculated with frozen culture directly from a 25% (w/v)

10   glycerol stock. The shake flask was incubated at 33°C and 175 rpm for approximately
     16 hours, whereupon 50 mL was used to inoculate the fermentor.

Fermentations

The fermentations were carried out in 6 L in house built fermentors.

15   The batch medium (3 L) contained:

Corn steep liquor (50% dw)	40 g/L
Yeast extract BioSpringer 153 (50% dw)	10 g/L
NaCl	5 g/L
$CaCl_2$, $2H_2O$	0.25 g/L
$Mn(NO_3)_2$, H_2O	0.2 g/L
Antifoam SIN260	1 mL/L
Kanamycin (filter sterilised to the fermentor after autoclaving	50 mg/L

20

The feed contained:

25

Glucose monohydrate	540 g/kg
$MgSO_4$, $7H_2O$	4.8 g/kg
Antofoam SIN260	4 mL/kg
Yeast extract, BioSpringer 153 (50% dw) (autoclaved separately)	150 g/kg

30

The feed in fermentation BAC0318 and BAC0323 was started based on the accumulated CO_2, according to the equations below:

$$Feed-flow[g/h] = 0, AcCO_2 < 0.15$$
$$Feed-flow[g/h] = 2.85 + t \cdot 1.54, AcCO_2 \geq 0.15 \text{ and } t < 12$$
$$Feed-flow[g/h] = 21.3, t > 12$$

5   t: time (hours) from the point when the accumulated CO_2 ($AcCO_2$) reached 0.15 moles.

The feed in fermentation BAC0319 and BAC0324 was started based on the accumulated CO_2, according to the equations below:

$$Feed-flow[g/h] = 0, AcCO_2 < 0.15$$
$$Feed-flow[g/h] = 2.0 + t \cdot 1.08, AcCO_2 \geq 0.15 \text{ and } t < 12$$
10
$$Feed-flow[g/h] = 15, t > 12$$

t: time (hours) from the point when the accumulated CO_2 ($AcCO_2$) reached 0.15 moles.

The pH was controlled at 7.0 by adding 12.5% (w/v) NH_3-water or 2M phosphoric acid.

15   The aeration was 3 L/min corresponding to 1 vvm.

The temperature was 33°C.

The fermentor was equipped with two 8 cm Ø Rushton impellers placed with a distance of 10 cm.

20   **Harvest**

The biomass was removed by centrifugation at 16,000× g for 10 minutes at room temperature. The supernatant was filter sterilized, and the filtrate was used for purification and application tests.

25

EXAMPLE 6. Application tests in egg yolk.

In the following experiments the isolated transferase from *Aeromonas salmonicida*
expressed in *E-coli* was tested in egg yolk alone and in egg yolk where a plant sterol

5 had been added.

Material

Transferase from *Aeromonas salmonicida* REF#138
10 Egg yolk: from fresh egg (hens eggs)
Plant sterol: β-sitosterol, Sigma S 5753

TLC plates: Silica plates Merck nr. 1.05715.0001

15 TLC analysis.
TLC-plate was activated in a heat cupboard (110°C) for ½ h.

100 ml developing solvent was poured into a chromatography camber with lid. The
walls of the chamber were covered with filter paper (Whatman 2) in order to saturate
20 the chamber with the solvent vapor.

The TLC-plate was placed in a frame and the sample was applied onto the TLC plate 2
cm from the bottom. The TLC plate was then placed in the TLC chamber with the
developing solvent. When the developing solvent reached 14 cm from the bottom of
25 the plate. The TLC plate was taken out and dried in fume board, and then placed in the
heat cupboard at 110 °C for 10 minutes.

The TLC-plate was then immersed in the developing reagent, and dried in the heat
cupboard at 110 °C for 15 minutes
30
Developing solvent:
Nr. IV: Chloroform : Methanol : H_2O (65:25:4)

Nr. I : P-ether : MTBE : Acetic acid (60:40:1)

Developing Buffer (Vanadate-buffer):

32 g Na$_2$CO$_3$ ad 300 ml H$_2$O (1M)

18.2 g vanadate pentoxide (V$_2$O$_5$) is added and dissolved during gentle heating.

The solution is cooled to ambient.

Carefully 460 ml 2.5 M H$_2$SO$_4$. (460 ml H$_2$O +61 ml H$_2$SO$_4$) is added

Water is added to 1000 ml.

Phospholipase activity.

Substrate:

0.6% L-α Phosphatidylcholine 95% Plant (Avanti #441601) + 0.4% Triton-X 100(

Sigma X-100) + 5 mM CaCl$_2$ is dissolved in 0.05M HEPES buffer pH 7.

Procedure.

400 µl substrate was added to an 1.5 ml Eppendorf tube and placed in a Eppendorf

thermomixer at 30°C for 5 minutes.

To the time T= 0   50µl enzyme solution was added. Also a blank with water instead of

enzyme was analysed.

The sample was mixed at 1000 rpm on Eppendorf Termomixer at 30°C for 10 minutes.

To the time T=10 min. The Eppendorf tube was placed in another termomixer at 99°C

for 10 minutes to stops the reaction.

Free fatty acid in the samples were analyzed by using the NEFA kit from WAKO

GmbH.

Enzyme activity PLU-7 pH 7 was calculated as micromole fatty acid produced per

minute under assay conditions.

Lipid extraction.

1 g egg yolk and 7.5 ml Chloroform:Methanol 2:1 was mixed on a Whirley and centrifuged at 750 x g for 10 minutes.

5   3ml of the chloroform phase was isolated and used for further lipid analysis..

Results:

The transferase (REF#138), from *Aeromonas salmonicida* expressed in *E-coli* was analysed for phospholipase activity as described above, and was

10   also tested in egg yolk with and without β-sitosterol. The sample was stirred with a magnetic stirrer during the reaction. The experimental design is shown in Table 1

Table 1

Test	Reaction time at 37°C	Egg yolk	Sitosterol	Transferase #138
Nr	Minutes	gram	mg	Units
1	30	1	40	
2	30	1	40	0,75 PLU
3	30	1	80	0,75 PLU
4	120	1	40	0,75 PLU
5	120	1	80	0,75 PLU
6	300	1	40	0,75 PLU
8	300	1	40	

15

The reaction was stopped by adding 7.5 ml Chloroform:Methanol (2:1) and mixed on a Whirley mixer for 30 seconds. The chloroform phase was isolated by centrifugation and 2 µl of the chloroform phase was transferred to a pre-activated silica TLC plate and eluted with developing solvent nr. I, and another TLC-plate in developing solvent

20   IV.

The results from the TLC analysis are shown in Figures 45 and 46.

Transferase reaction with a transferase from *Aeromonas salmonicida* in egg yolk where plant sterol was added has shown that the enzyme transfers fatty acid from
5 lecithin in the egg yolk to the cholesterol during formation of cholesterolester. The TLC chromatogram also indicated that part of the sterol added to egg yolk was transferred to sterol ester.

The amount of sterol ester relative to the amount of cholesterol ester formed during the
10 reaction can be analysed by HPLC or GLC.

It is known that plant sterol esters reduce the absorption of cholesterol in the intestine. It is also indicated in the literature that cholesterolesters are absorbed less than free cholesterol in the intestine. When a transferase and plant sterol is added to egg yolk a
15 product with causes reduced cholesterol absorption is obtained, and at the same time lysolecithin is produced which improves the emulsification properties of the egg yolk. A further advantage of adding transferase and plant sterol to egg yolk is that plant sterol ester is ingested together with the natural available cholesterol, which is expected to have the highest effect on the reduction of cholesterol absorption.

20

EXAMPLE 7: Modification of egg yolk by lipid acyl transferase from *Aeromonas salmonicida.*

In accordance with the present invention it has now been shown that it is possible to
25 produce lysolecithin from egg yolk without substantial free fatty acid formation by use of a transferase.

The lecithin content of egg yolk is an important emulsifier for the production of mayonnaise with the limitation that the mayonnaise is not heat stable. It has therefore
30 been known for several years to use a phospholipase from pancreas to modify lecithin in egg yolk to lysolecithin, which is a more efficient emulsifier. The use of enzyme modified egg yolk in mayonnaise production contributes to better heat stability of the

mayonnaise during pasteurisation. A limitation of using pancreas phospholipase in egg yolk is that the amount of free fatty acid also increases, which contributes to reduced oxidative stability because free fatty acids are more prone to oxidation than the corresponding ester. Free fatty acid may also contribute to a soapy off taste.

5

The transferase from *Aeromonas salmonicida* was successfully expressed in *B. subtilis* and fermented in lab scale as described in Example 5, purified by liquid chromatography and used to modify egg yolk lipids. The enzyme modified egg yolk was used to produce heat stable mayonnaise.

10

The transferase from *A. salmonicida* can be used to produce lysolecithin and cholesterolester in egg yolk without production of significant amounts of free fatty acids. That is to say without increasing or substantially increasing the free fatty acids in the foodstuff.

15

The enzyme modified egg yolk produced by transferase showed improved emulsification properties and can be used for heat stable mayonnaise.

This enzyme was highly functional in modification of egg yolk by catalysing the lipid
20   transfer reaction between lecithin and cholesterol Figure 47.

This study further investigated the use of transferase for modification of egg yolk and the use of modified egg yolk in the production of heat stable mayonnaise.

25   This example describes the fermentation, isolation, and application of the transferase in egg yolks as well as the application of the enzyme modified egg yolk in mayonnaise. The example is divided into two parts:

A. Application of transferase in egg yolk
30   B. Testing of enzyme modified egg yolk in mayonnaise

EXPERIMENTAL

A. Application

5 *Enzyme and substrate*

Transferase #178-9 from *A. salmonicida*, purification 2554-100 C73, 15 PLU-7/ml.

Transferase #179 from *A. salmonicida*, 18.5 PLU-7/ml.

Phospholipase A1 LECITASE™ Ultra (Novozymes A/S, Denamrk)

Egg yolk: Liquid egg yolk with 8% salt, SANOVA FOODS, DK

10

TLC analysis was performed as described previously (see above Example 6).

Phospholipase activity: See previous examples.

15 *Lipid extraction*

1 g egg yolk and 7.5 ml Chloroform:Methanol 2:1 was mixed on a Whirley for 30 sec. and centrifuged at 750 x g for 10 minutes.

4 ml of the chloroform phase was isolated and used for further lipid analysis.

20 **Oxidation stability test**

Oxidation stability of mayonnaise was measured in an ML OXIPRESS equipment where the sample is oxidative stressed by means of heat under pressure in an oxygen atmosphere.

After a certain time, called the induction period (IP), the oxidation of the sample

25 causes a certain consumption of oxygen, which is registered as pressure change of a pressure transducer. Higher induction period indicates better oxidation stability.

Procedure.

5-gram mayonnaise is placed in a glass container and the glass container is closed with

30 the pressure transducer. The container is filled with oxygen to 5 bars. The valve is opened to empty the container. This procedure is repeated twice and the sample with 5

bar oxygen atmosphere is placed at 80 °C. The oxygen pressure as a function of time is measured and the induction period (IP) calculated in hours.

Results

5   Purified transferase from Aeromonas salmonicide sample no. #179 and #178-9 were used to treat egg yolk as outlined in Table 2. The initial test has shown that GCAT transferase should be added with much lower phospholipase (PLU) activity, than a commercial Phospholipase. This is explained by the fact that GCAT is a transferase and therefore has much lower hydrolytic activity than a normal phospholipase.

10

Table 2

nr	Sanofo egg yolk 8%salt Egg yolk	2344-44 C89 18,5 PLU-7/ml Transferase #179	Transferase #178-9 18.5 PLU-7/ml	# 3108, Lecitase Ultra 1500 PLU-7/ml 7/ml	Water	
	gram	gram	gram	ml	gram	PLU-7/ml
6	120	2.00			8.00	0.31
7	120		10		0	1.25
8	120			1.86	8.14	23.25
9	120				10	0

The enzymatic reactions were conducted by scaling the egg yolk and the enzyme in a beaker. The samples were placed in a heating cabinet at 37 °C during slow agitation.

15   After 1, 2 and 4 hours reaction time a sample was taken out for TLC analysis. After 4 hours reaction time the product was stored at 5 °C and used for mayonnaise experiments.

The TLC analyses of lipids extracted from enzyme treated egg yolk is shown in Figure

20   48.

The TLC analysis in Figure 48 shows a clear hydrolytic effect of Phospholipase #3108 on triglyceride during formation of free fatty acids, as well as some mono- and diglyceride. Phospholipase #3108 seem to have no effect on cholesterol. Both transferase samples clearly contribute to the formation of cholesterol ester concomitant with the reduction of the cholesterol content.

D. Enzyme modified egg yolk in Mayonnaise

In order to investigate the effect of the modification of the egg yolk samples mentioned in Table 2, application trials were performed on mayonnaise with a fat content of 50%. A mayonnaise containing untreated egg yolk was also produced.

The aim of the investigation was to determine the impact of enzymatically modified egg yolks' emulsification properties and the impact on heat stability. All mayonnaise samples contained the same oil level and were emulsified with only egg yolk.
The mayonnaise samples were all produced using a Koruma mixer (Disho V60/10) and heated during processing to 95°C for 5 minutes.

Samples of the mayonnaises (Figure 49) produced by enzyme treated egg yolk were nice and homogenous with no oil separation. The control sample separated in an oil and a water phase.

The particle size of oil droplet in the mayonnaise samples with enzyme treated egg yolk was measured on a Malvern Mastersizer. The sample was mixed with 0.1%SDS in 0.1 M phosphate buffer pH 7 prior to measurement. Reading was mean size of all particles as shown in Table 3.

Table 3.

Experiment	Enzyme	Mean particle size, μm
6	Transferase #179, 0.31 PLU-7/g	12.9
7	Transferase #178-9, 1.25 PLU-7/g	7.2
8	#3108, Lecitase Ultra, 23 PLU-7/g	5.2

The results from the particle size measurement clearly show the effect of increased
dosage of transferase from *A. salmonicida*. With the high dosage of transferase the
particle size is close to the mayonnaise produced by Lecitase Ultra. It should however
be kept in mind that Lecitase Ultra produces a lot of fatty acids, which might
contribute to a finer particle distribution.

The oil droplet size of the mayonnaise prepared with the enzyme is significantly
smaller than the oil droplet size of the mayonnaise prepared without the enzyme (i.e.
the control mayonnaise).

Oxidation stability

The oxidation stability of the mayonnaise samples 7 and 8 were analyzed on a ML
OXIPRES with results shown in Table 4.

Table 4

Sample	Induction period	Induction period
	1. determination hours	2. determination hours
7	37.44	38.08
8	35.68	35.52

Measurement of oxidation stability gave a clear significant difference in oxidation stability. The mayonnaise with transferase 179-8 treated egg yolk had a significant better oxidation stability than the mayonnaise with Lecitase Ultra treated egg yolk. This might be explained by the fact that Lecitase Ultra produces more free fatty acids

5   which are more prone to oxidation that the corresponding fatty acid esters.

A sample of the egg yolks used for mayonnaise production were extracted with chloroform, and the lipids from the egg yolk were analysed by GLC with results

10   shown in Table 5.

Table 5

Experiment	Enzyme	Fatty acid	Cholesterol	Cholesterol ester	Triglyceride
6	Transferase #179	0.96	0.94	0.49	23.95
7	Transferase #178-9	1.84	0.60	1.06	24.54
8	#3108, Lecitase Ultra	14.05	1.16	0.12	2.45
9	Control	0.48	1.16	0.13	22.87

15   The GLC results in Table 5 confirm the results form the TLC analysis that Lecitase Ultra produces a very high amount of free fatty acids and a large part of the triglyceride is hydrolysed. On the other hand the transferase produces only modest amount of free fatty acids and no triglycerides are hydrolysed. It is also clearly shown that transferase produce cholesterol ester from cholesterol.

20

The results indicate that the amount of PC in the "enzyme treated" mayonnaise is reduced as compared with the control mayonnaise, whilst the amount of LPC is increase in the enzyme treated mayonnaise as compared with the control mayonnaise.

25   The increase in the amount of LPC may well explain the improved emulsification

properties of the enzyme treated mayonnaise as compared with the control mayonnaise. The HPLC and GLC analyses also indicate a lower level of free cholesterol in the enzyme treated mayonnaise as compared with the control mayonnaise, probably due to the cholesterol being used as an acceptor molecule in the

5 transferase reaction resulting in an increase in the amount of cholesterol esters in the enzyme treated mayonnaise as compared with the control mayonnaise. In addition, the results indicate that the amount of free fatty acids do not increases significantly when egg yolk is treated with the transferase. The results further indicate that the amount of free fatty acids produced in the foodstuff treated with the lipid acyltransferase is

10 significantly lower than in the foodstuff treated with the control phospholipase, this is true even if the amount of lysolecithin formed in the foodstuffs is the same.

EXAMPLE 8: Effect of *Aeromonas salmonicida* transferase in cakes.

15 The effect of GCAT acyl-transferase form *Aeromonas salmonicida* is tested in a cake recipe. The enzyme is tested alone and in combination with other lipolytic enzymes. The enzymes are added to some of the cake ingredients or added together with the other cake ingredients before mixing the cake.

20 Preliminary results show that acyl-transferase combined with a triglyceride-hydrolysing enzyme improves the cake volume and crumb structure compared with a control.

In the following experiments a transferase from *A. salmonicida* and variants are tested

25 alone and in combination with triglyceride hydrolysing enzymes. These enzymes are active on the lipid components in the egg and the shortening as well as on the carbohydrates, protein, glycerol and cholesterol (in egg), which forms part of the cake recipe.

30

Materials and method

Enzyme

#179, Acyl-transferase from *Aeromonas salmonicida*

5   Grindamyl EXEL 16, Lipase from *Thermomyces lanuginisus*

Cake recipe:

Ingredients	%	g
Sugar 35/20	20,37	204
Cake flour, Albatros	18,11	181
Wheat starch	5,21	52
Baking powder	0,36	4
Pasteurised liquid whole egg	22,63	226
Shortening Vegao (Aarhus United)	18,11	181
Whey powder	0,68	7
Glucose sirup ,75% 42 DE	4,53	45
Glycerol	1,36	14
Salt	0,32	3
Rape seed oil	6,34	63
Potassium sorbate	0,18	1,8

10   Equipment:

Mixer: Hobart N50 with a spatula

Oven: Simon cake oven

15

Procedure:

All ingredients must be tempered to room temperature.

1. Cream up sugar and shortening for 3 minutes – start at 2nd speed and move to 3rd speed within 30 sec.

2. Add remaining ingredients – start at 1st speed and move to 2nd speed within 30 sec – mix total 5 min

3. Measure the volume of the batter in 1 dl cup

4. The pound cake tins are sprayed with "Babette" oil spread, and covered with paper

5. Scale 2 x 350 g into the pound cake tins

6. Spread out the mass evenly with a spatula

7. Before put in the oven – add a string of oil on top of the cake (lengthwise in the middle – to make the cake break in the middle

8. Bake for 50 min. at 180°C

9. After baking take the tins out of the oven, and "drop" it on the table, before taking the cakes out of the tins

10. Take paper off the cakes and turn the right side up

11. The cakes are cooled on a grating for 60 min. before weighing and measuring of the volume

Remarks:

The enzyme(s) used is/are added at the beginning of mixing or is/are added to some of the cake ingredients before added to the other cake ingredients.

The enzymes are only active during the mixing or reaction of cake components, and the enzymes are inactivated during baking of the cake.

Results.

The following experiments are conducted as shown in the following table:

		1	2	3.	4
Whole egg	g	250	250	250	250
Glucose syrup, 75% DE 42	g	10	10	10	10
#179 acyl-transferase, 26 PLU/ml	ml	25		25	
Grindamyl EXEL 16,	mg		37,5	37,5	
Water					25

Egg, Glucose syrup and enzyme are reacted for 30 minutes at 37 °C and shortly after
5 the eggs are use to produce cake according to the recipe mentioned above.

Preliminary results show that a combination of acyltransferase and a triglyceride
hydrolysing lipase from *Thermomyces lanoginosus* improves the cake volume, and
also the crumb structure, eating quality and appearance is improved compared with a
water control. Preliminary results indicate in cake it may be preferably to use a
10 combination of lipid acyltransferase and a lipase.

EXAMPLE 9: The purpose of these experiments was to test a transferase from *A. hydrophila* expressed in *E. coli.*

15 The transferase reaction of *A. hydrophila* #135 (0.5 NEFA-PLU/ml) was tested in egg
yolk. The experimental set-up is shown in Table 6.

Table 6

	Reaction time	Egg yolk	#135 conc.
Nr	Minutes	Gram	Units, PLU-NEFA
1	30	1	0,000
2	30	2	0,100
3	60	2	0,100

4.	150	2	0,100
5	240	2	0,100
6	1560	2	0,100
7	1560	1	0,000

The egg yolk was heated to 37°C and the enzyme added. After reaction time 7 ml CHCl₃:Methanol 2:1 was added and mixed on a Whirley for 30 sec.

5   The sample was centrifuged 800 x g for 10 minutes and the lower solvent phase isolated. 2 µl of this sample was applied onto a TLC Silica plate and eluted in elution solvent IV. The results from the TLC analysis is shown in Figures 50 and 51.

The methods and materials mentioned in this Example are those detailed in Examples

10   above.

Samples from this experiment was also analysed by GLC as TMS derivatives. The results from the GLC analysis are shown in Table 7.

15   Table 7. GLC analysis of lipid from egg yolk

No.	Reaction	Transferase #135 conc.			
	time	Units/g egg yolk	Free fatty acid	Cholesterol	Cholesterolester
	min		%	%	%
7	control	0	0,25	2,88	0,34
3	60	0,025	0,25	2,68	0,56
4	150	0,025	0,29	1,85	1,72
5	240	0,025	0,53	1,42	3,54
6	1560	0,025	0,95	0,3	4,43

From the GLC analysis of free fatty acid, cholesterol and cholesterolester it is possible to calculate the molar concentration of each component and calculate % transferase activity as shown in Table 7.

5   Calculation of % transferase activity

From the results the increase in free fatty acid, sterol esters are calculated

Δ % fatty acid = % Fatty acid(enzyme) - % fatty acid(control)

Δ % sterol ester = % sterol/stanol ester(enzyme) - % sterol/stanol ester(control)

10   The transferase activity is calculated as % of the total enzymatic activity:

% transferase activity=

$$\frac{(\Delta \% \text{ sterol ester}/(\text{Mv sterol ester}) \times 100}{\Delta \% \text{ sterol ester}/(\text{Mv sterol ester}) + \Delta \% \text{ fatty acid}/(\text{Mv fatty acid})}$$

15

where:

Mv sterol ester = average molecular weight of the sterol esters
Mv fatty acid   = average molecular weight of the fatty acids

20

Table 8 Transferase activity in egg yolk of *A.hydrophila* #135

No.	Reaction	Transferase #135 conc.				
	Time	Units/g egg yolk	Free fatty acid	Cholesterol	Cholesterol-ester	Transferase activity
	min		mM	mM	mM	%
7	Control	0	8,9	74,5	5,3	-
3	60	0,05	8,9	69,3	8,7	100
4	150	0,05	10,4	47,8	26,5	93
5	240	0,05	18,9	36,7	54,6	77
6	1560	0,05	33,9	7,8	68,4	48

Both TLC and GLC analysis confirm that initially the transferase reaction of *A. hydrophila* #135 is the dominating reaction. After 150 minutes reaction time some
5 hydrolytic activity occurs. After 1560 minutes the transferase reaction and the hydrolytic reaction has almost reached the same level. The results also indicate that as long as the acceptor molecule cholesterol is available the transferase reaction is the dominating reaction. When the concentration of cholesterol decreases the hydrolytic activity becomes more dominant.
10

EXAMPLE 10: Assay for measurement of transferase activity using egg yolk as substrate – hereinafter referred to as the "Egg Yolk Assay"

A lipid acyltransferase was isolated from *Aeromonas salmonicida* and expressed in
15 *Bacillus subtilis*. The purpose of this work is to develop an analytical method, which is
· able to .
measure both transferase and hydrolytic activity of enzymes and from these analyses it is possible to define both transferase and hydrolytic activity of enzymes using a substrate which contain lecithin and cholesterol.
20

In this work egg yolk was used as substrate for the enzyme assay because egg yolk contain both lecithin and cholesterol and it is known that transferases and phospholipases works very well in this substrate.

25 The drawback by using egg yolk is that this substrate is a complex mixture of water, lipids, and proteins. Lipid components include glycerides, 66.2%; phospholipids, 29.6%; and cholesterol, 4.2%. The phospholipids consist of 73% lecithin, 15% cephalin , and 12% other phospholipids. Of the fatty acids, 33% are saturated and 67% unsaturated, including 42% oleic acid and 7% linoleic acid (ref. Kirk-Othmer
30 Encyclopedia of Chemical Technology, John Wiley & Sons, Inc.)

Some variations in the egg yolk composition might be expected. In the literature (Biochimica et Biophysica Acta, 1124 (1992) 205-222) it is however mentioned that "The mature egg yolk of the domestic hen possesses remarkably constant lipid and lipoprotein composition despite much variation in dietary and environmental conditions", and further it is quoted "As a result the egg yolk continues to provide a food product of nearly constant composition, which serves to maintain its chemical and physical-chemical properties for reliable utilization in the baking, cosmetic and pharmaceutical industries"

This reference indicates that egg yolk composition is very constant and it was therefore decided to use hens egg yolk as substrate for the Egg Yolk Assay.

Quantification of lipid reaction products from enzymatic treatment of egg yolk was made by extraction of lipids from the substrate followed by GLC analysis of the lipid components.

Procedure

Materials.

Egg yolk: Pasteurised liquid egg yolk from Danæg Products A/S, DK- 4000 Roskilde.
HEPES buffer Sigma cat. no. H 3375
Chloroform, Analytical grade

Enzymes.

Purified lipid acyltransferase from *A. salmonicida* #178-9

Thermomyces lanuginosus lipase. GRINDAMYL EXEL 16 , item nr. 147060 (Control)

Enzyme assay with egg yolk substrate.

5 gram liquid egg yolk was scaled in a 20ml Wheaton glass and heated to 35 °C
0.25ml enzyme solution was added and a stopwatch is started.

At regular intervals 0.5g samples were transferred to a 10ml Dram glass.

20 µl 4M HCl was added in order to stop the enzyme reaction and acidify the fatty acid soap.

3 ml Chloroform was added. And the sample was mixed on a Whirley mixer for 30 sec.

The sample was centrifuged at 3000 g for 10 min and 0.8 ml of the chloroform phase was transferred to a tarred Dram glass. Chloroform was evaporated at 60 °C under a steam of nitrogen. The dram glass was scaled again.

The isolated lipids were analysed by GLC and TLC.

TLC analysis – as previously described.

GLC analysis – as previously described.

Results

For the Egg Yolk Assay using egg yolk as substrate the experiment shown in Table 9 was conducted.

Table 9

		1	2	3
Egg yolk, liquid.	gram	5	5	5
Transferase# 178-9, 32 PLU-7/ml*	ml			0.25
T.lanuginosus lipase, 200 LIPU/ml	ml		0.25	
Water	ml	0.25		

0.5g samples were taken out after 15, 30, 60 120 and 1080 minutes, and the lipid isolated by solvent extraction. The lipids were analysed by TLC using solvent I and IV respectively. Picture of the TLC plate is shown in Figure 52.

The TLC analysis clearly indicates the activity of transferase #178-9 from *A. salmonicida* (sample 3). This can be seen from the decrease in the phospholipids PC and PE. The results also indicate that the amount of lysolecithin LPC is not as high as expected. This might indicate hydrolytic activity on lysolecithin or it might also be
5   caused by insufficient extraction because lysolecithin is very polar and therefore could be partly distributed in the water phase.

The lipids isolated by solvent extraction was also analysed by GLC in order to quantify the amount of free fatty acid, cholesterol and cholesterol ester. The GLC
10   results are shown in Table 10.

Table 10. GLC analysis of lipid from enzyme treated egg yolk. Results are in % based on lipid content.

			15 Minutes	30 Minutes	60 Minutes	120 Minutes	1080 Minutes
Free fatty acids	Control	1	0.328	0.304	0.332	0.333	0.369
	T. lanuginosus	2	0.391	0.376	0.459	0.627	22.909
	A. salmonicida #178-9	3	1.007	1.668	4.013	6.761	15.098
Cholesterol	Control	1	3.075	2.968	3.103	3.056	3.099
	T. lanuginosus	2	3.130	3.032	3.045	3.026	3.225
	A. salmonicida #178-9	3	2.835	1.912	0.356	0.220	0.206
Cholesterol ester	Control	1	0.416	0.397	0.422	0.408	0.437
	T. lanuginosus	2	0.436	0.400	0.425	0.419	0.416
	A. salmonicida #178-9	3	1.414	2.988	6.107	6.694	5.760
Triglyceride	Control	1	76.153	73.505	75.565	79.344	77.382
	T. lanuginosus	2	74.099	74.413	77.079	74.284	21.781
	A. salmonicida #178-9	3	73.781	73.342	77.857	82.040	72.117

From the results it was observed that almost all the cholesterol was esterified after 60 minutes in sample 3. It was concluded that for the first 30 minutes there was surplus

5   substrate for the reaction. The results form samples taken out after 15 and 30 minutes were therefore used to calculate the activities of the enzymes.

Based on the information in table 10 and the fact that egg yolk contain 27% lipid the amount of micromole fatty acid and cholesterol ester produced per ml enzyme was

10   calculated with results shown in Table 11

The results in Table 11 were obtained be the following calculations of the results from fatty acids in sample no.3 (*A. salmonicida*, 15 min.)

15   Lipid in 5 g egg yolk = 5*0.27 = 1.35 gram

1.35 gram lipid contain 1.007% fatty acids = 1.35*1.007/100 = 0.01359 gram

Average molecular weight of fatty acids is 272

0.01359gram = 0.01359*1000000/272 μmol = 49.9798 μmol

0.25 ml enzyme is added

20   μmol Fatty acid/ml enzyme = 49.9798/0.25 = 199.9

Table 11

Micromole/ml enzyme				
		0 min	15 min	30 min
Free fatty acid	Control		65.01	60.37
	T. lanuginosa		77.61	74.71
	Transferase #178-9		199.86	331.06
Cholesterol ester	Control		35.09	33.50
	T. lanuginosa		36.77	33.73

			119.29	252.15
	Transf. #178-9		119.29	252.15

From the results in Table 11 it is possible to calculate the change in amount of fatty acid and cholesterol ester caused by the enzyme relative to control as shown in Table 12.

5   Table 12.

Δ Micromole/ml enzyme		0 min	15 min	30 min
Free fatty acid	T. lanuginosus	0	12.593	14.340
	Transf. #178-9	0	134.843	270.691
Cholesterol ester	T. lanuginosus	0	1.677	0.235
	Transf. #178-9	0	84.196	218.652

The amount of fatty acid or cholesterol ester produced as a function of time is shown in Figure 53.

10   From the slope of the curve the hydrolytic activity (FFA formation) and the lipid acyltransferase activity (cholesterol ester formation) as a function of time was calculated. The relative transferase activity (% acyltransferase activity) and the relative hydrolytic activity were then calculated as shown in Table 13. The relative transferase activity was determined using the protocol for the determination of % acyltransferase

15   activity as described hereinbefore. For example, calculation of relative activity for #178-9:Total activity is FFA activity + transferase activity = 9,023+7,2884= 16,311 µmol/min/ml, Relative transferase activity= 7,2884*100/16,311=44,7, Relative hydrolytic activity= 9,023*100/16,311= 55,3

Table 13.

T. lanuginosus	FFA activity	0.4780	µmol/min/ml
A. salmonicida #178-9	FFA activity	9.0230	µmol/min/ml

T. lanuginosus	Cholesterol ester. Activity	0.0078	μmol/min/ml
A. salmonicida #178-9	Cholesterol ester. Activity	7.2884	μmol/min/ml
T. lanuginosus	Relative transferase activity	1.6	
A. salmonicida #178-9		44.7	
T. lanuginosus	Relative hydrolytic activity	98.4	
A. salmonicida #178-9		55.3	

The results in Table 13 confirmed that the transferase enzyme from *A. salmonicida* has a significant transferase activity, but the results also confirmed that this enzyme has a significant hydrolytic activity.

5

The lipase from T. lanuginosus mainly has hydrolytic activity, and the relative transferase activity 1.6 was not a proof of any transferase activity but was explained by the uncertainty of the analysis.

10   Conclusion.

Egg yolk was used as substrate for the measurement of transferase and hydrolase activity of lipid acyltransferase from *Aeromonas salmonicida* and a lipase from *Thermomyces lanuginosus*. Under assay conditions there was initially a linear relation between cholestererol ester and free fatty acid formation and time for the lipid

15   acyltransferase enzyme. Based on this linear relationship it was possible to calculate the hydrolytic activity (FFA formation) and the transferase activity (cholesterol ester formation). The relative hydrolytic and transferase activity was also calculated. The lipid acyltransferase (in this case a GCAT) from *Aeromonas salmonicida* showed almost equal hydrolytic and transferase activity under these assay conditions.

20   Lipase from *Thermomyces lanuginosus* showed very low hydrolytic activity and the transferase activity was not significant.

EXAMPLE 11: Transferase Assay in High Water Egg Yolk.

Introduction

5

A lipid acyltransferase in accordance with the present invention was isolated from *Aeromonas salmonicida* and expressed in *Bacillus subtilis*. Initial experiments have shown that this enzyme is very efficient in transferring fatty acid from lecithin to cholesterol using egg yolk as a substrate.

10   In the following experiments the transferase reaction was studied in further detail using egg yolk as a substrate with special focus on the water concentration in the substrate.

Procedure

15

Materials.

Egg yolk: Pasteurised liquid egg yolk from Danæg Products A/S, DK- 4000 Roskilde.

HEPES buffer Sigma cat. no. H 3375

Chloroform, Analytical grade

20   Squalane, analytival grade

Enzymes.

#178-9 Lipid acyl transferase in accordance with present invention from *A. salmonicida*

25   #2427 Phospholipase A1 from *Fusarium oxysporum*. LIPOPAN® F from Novozymes, DK (comparative lipolytic enzyme)

#1991 Phospholipase A2 from Pancreas, LIPOMOD 22L from Biocatalysts, UK (comparative lipolytic enzyme)

30

Enzyme assay with egg yolk substrate.

5 gram liquid egg yolk substrate was scaled in a 20ml Wheaton glass and heated to 35 °C

Water and enzyme solution was added and a stopwatch is started.

At regular intervals 0.5g samples was transferred to a 10ml Dram glass.

5 20 µl 4M HCl was added in order to stop the enzyme reaction and acidify the fatty acid soap.

3 ml Chloroform was added. And the sample was mixed on a Whirley mixer for 30 sec.

The sample was centrifuged at 3000 g for 10 min and 0.8 ml of the chloroform phase

10 was transferred to a tarred Dram glass. Chloroform was evaporated at 60 °C under a steam of nitrogen. The dram glass is scaled again.

The isolated lipids are analysed by GLC

GLC analysis

15

Perkin Elmer Autosystem 9000 Capillary Gas Chromatograph equipped with WCOT fused silica column 12.5 m x 0.25 mm ID x 0.1 µ film thickness 5% phenyl-methyl-silicone (CP Sil 8 CB from Chrompack).

Carrier gas: Helium.

20 Injector. PSSI cold split injection (initial temp 50°C heated to 385°C), volume 1.0µl

Detector FID: 395°C

Oven program:	1	2	3
Oven temperature, °C.	90	280	350
Isothermal, time, min.	1	0	10
25 Temperature rate, °C/min.	15	4	

Sample preparation: 30 mg of sample was dissolved in 9 ml Heptane:Pyridin, 2:1 containing internal standard heptadecane, 0.5 mg/ml. 300µl sample solution was transferred to a crimp vial, 300 µl MSTFA (N-Methyl-N-trimethylsilyl-

30 trifluoraceamid) was added and reacted for 20 minutes at 60°C.

Calculation: Response factors for mono-di-triglycerides and free fatty acid were determined from Standard 2 (mono-di-triglyceride), for Cholesterol, Cholesteryl

palmitate and Cholesteryl stearate the response factors were determined from pure reference material (weighing for pure material 10mg).

Results

Egg yolk containing 2% squalane was used as substrate for the reactions. Squalane was added as an internal standard for the GLC analysis, in order to quantify the lipid components in egg yolk.

The experiment was set up as shown in Table 14.

Table 14.

		1	2	3	4	5	6	7	8
Substrate, egg yolk with 2%squalane	g	5	5	5	5	5	5	2.5	2.5
Transferase # 178-9 , 14 PLU-7/ml	ml		0.25			0.25		0.13	
LIPOPAN® Fsolution , 200 PLU-7/ml	ml			0.25					0.13
#1991 Phospholipase A2, 6300 PLU/ml	ml				0.25		0.25		
Water	ml	0.25				3.8	3.8	8.75	8.75

Samples were taken out after 30, 60 and 120 minutes and analysed according the method described above (0.5 ml (exp 1-4) 0.86 ml (exp. 5-6) and 2.2 ml(exp.7-8) samples were taken).

The results from the GLC analysis are shown in Table 15. The GLC results were expressed in percent of the substrate (egg yolk). The table also indicate the reaction time and the total amount of water in the reaction mixture.

Table 15.

Enzyme	Reaction time	Water %	GLC	GLC	GLC
	minutes	in reaction	% Fatty acid	% cholesterol	% cholesterol ester
Control	120	54	0,247	0,863	0,083
# 178	30	54	0,422	0,669	0,445
# 178	60	54	0,515	0,549	0,672
# 178	120	54	0,711	0,364	1,029
#2427	30	54	2,366	0,848	0,090
#2427	60	54	3,175	0,837	0,088
#2427	120	54	3,926	0,833	0,082
#1991	30	54	1,606	0,911	0,083
#1991	60	54	1,701	0,838	0,080
#1991	120	54	1,781	0,763	0,053
# 178	30	73	0,377	0,764	0,495
# 178	60	73	0,488	0,665	0,719
# 178	120	73	0,626	0,426	0,931
#2427	30	73	2,471	0,853	0,092
#2427	60	73	3,284	0,858	0,087
#2427	120	73	4,176	0,837	0,081
# 178	30	89	0,344	0,720	0,308
# 178	60	89	0,443	0,725	0,446
# 178	120	89	0,610	0,597	0,607
#2427	30	89	0,510	0,167	0,010
#2427	60	89	0,602	0,133	0,010
#2427	120	89	0,867	0,147	0,009

Based on the analyses of fatty acid, cholesterol and cholesterol ester it was possible to
5   calculate the amount of free fatty acid, and cholesterol ester produced as a function of
reaction time and water content. Based on these results it was then possible to calculate
the total enzymatic activity as the sum of the fatty acid formation and the cholesterol

ester formation. The relative hydrolytic activity and the relative transferase activity (i.e. % acyltransferase activity) were then calculated with the results shown in Table 16.

5   The results in Table 16. were also analysed statistically using a Statgraphic Multifactor ANOVA . The statistical results in Figure 54 confirm that Phospholipase A1, #2427 and phospholipase A2, #1991 have no transferase activity whereas the transferase #178-9 showed almost 50% transferase activity under these assay conditions.

10

The effect of water content in the assay on the transferase activity of the transferase #178 was also analysed statistically as shown in Figure 55. These results indicate that in the range from 54 to 89% water in the assay there was no strong effect of the water content on the relative transferase activity.

15

The impact of reaction time on transferase activity for transferase #178 was evaluated with results shown in Table 16 and Figure 56. The results in Figure 56 indicate that the relative transferase activity decreases as a function of reaction time. This might be explained by the fact that most of the acceptor molecule cholesterol is consumed and 20   therefore the relative hydrolytic activity increases. The negative values for transferase reaction for #2427 only indicate no transferase activity within the variation for the analytical method.

Table 16.

Enzyme	Reaction time minutes	Water % in reaction mixture	Fatty acid produced	Cholesterol consumed	Cholesterol ester produced	Hydrolytic activity %	Transferase activity %
# 178	30	54	0,175	0,194	0,362	53	47
# 178	60	54	0,268	0,314	0,589	52	48
# 178	120	54	0,464	0,499	0,946	53	47

#2427	30	54	2,119	0,015	0,007	100	0
#2427	120	54	2,928	0,026	0,005	100	0
#2427	60	54	3,679	0,030	-0,001	100	0
#1991	30	54	1,359	-0,048	0,000	100	0
#1991	60	54	1,454	0,025	-0,003	100	0
#1991	120	54	1,534	0,100	-0,030	101	-1
# 178	30	73	0,130	0,099	0,412	42	58
# 178	60	73	0,241	0,198	0,636	47	53
# 178	120	73	0,379	0,437	0,848	51	49
#2427	30	73	2,224	0,010	0,009	100	0
#2427	60	73	3,037	0,005	0,004	100	0
#2427	120	73	3,929	0,026	-0,002	100	0
# 178	30	89	0,097	0,143	0,225	50	50
# 178	60	89	0,196	0,138	0,363	56	44
# 178	120	89	0,363	0,266	0,524	62	38
#2427	30	89	0,263	0,696	-0,073	113	-13
#2427	60	89	0,355	0,730	-0,073	110	-10
#2427	120	89	0,620	0,716	-0,074	105	-5

Conclusion.

5   The lipid acyltransferase from *Aeromonas salmonicida* was tested in egg yolk as substrate and with different levels of water content. This enzyme was compared with control lipolytic enzymes, namely Phospholipase A1 from *Fusarium oxysporum* and a *Phospholipase* A2 from pancreas.

10   The results have proved that only the transferase catalysed the transferase reaction between lecithin and cholesterol during formation of cholesterol ester. The results showed that in the range from 54% to 89 % water in the substrate the relative transferase activity was almost the same for transferase from *Aeromonas salmonicida.*

EXAMPLE 12 . : The "Transferase Assay in Buffered Substrate" for measurement of acyltransferase activity (e.g. for use in a foodstuff using lecithin and cholesterol).

The lipid acyltransferase was isolated from *Aeromonas salmonicida* and expressed in *Bacillus subtilis*. This enzyme is very efficient in transferring fatty acid from lecithin to cholesterol during formation of cholesterol esters. It has also been shown that the enzyme has some hydrolytic activity, which is observed by the formation of free fatty acid. Traditional phospholipases (EC3.1.1.4 and EC3.1.1.32) have the ability to hydrolyse lecithin during formation of free fatty acids and lysolecithin, and no transferase reactions has been reported for these enzymes.

We detail herein an assay that is able to measure both transferase and hydrolytic activity of enzymes and thus to identify lipid acyltransferases in accordance with the present invention, the assay uses a substrate which contains lecithin and cholesterol. In this work a substrate based on phosphatidylcholine and cholesterol dispersed in a buffer was used. Quantification of reaction products was made by extraction of lipids from the substrate followed by GLC analysis of the lipid components.

Procedure

Materials
L-alpha-Phosphatidylcholine 95% (Plant) Avanti no. 441601 .
Cholesterol: Sigma cat. C 8503
Cholesteryl Palmitate, Sigma C 6072
Cholesteryl Stearate, Sigma C 3549
HEPES buffer Sigma cat. No. H 3375
Chloroform, Analytical grade.

Enzymes
Purified GCAT from *A. salmonicida* #178-9

TLC analysis.

TLC-plate was activated in a heat cupboard (110°C) for ½ h.

100 ml running buffer was poured into a chromatography chamber with lid. The walls
5   of the chamber were covered with filter paper (Whatman 2) in order to saturate the
chamber with the solvent vapour.

The TLC-plate was placed in a frame and the sample was applied onto the TLC plate 2
cm from the bottom. The TLC plate was then placed in the TLC chamber with the
running buffer. When the running buffer reached 14 cm from the bottom of the plate,
10   the TLC plate was taken out and dried in fume board, and then placed in the heat
cupboard at 110 °C for 10 minutes.

The TLC-plate was then immersed in the developing reagent, and dried in the heat
cupboard at 110 °C for 15 minutes

15   Running-buffer:

Nr. IV: Chloroform : Methanol : H_2O (65:25:4)

Nr. I: P-ether : MTBE : Acetic acid (60:40:1)

20   Developing buffer (Vanadate-buffer):

32 g Na_2CO_3 ad 300 ml H_2O (1M)

18.2 g vanadate pentoxide (V_2O_5) is added and dissolved during gentle heating.

The solution is cooled to ambient.

Carefully 460 ml 2.5 M H_2SO_4. (460 ml H_2O +61 ml H_2SO_4) is added
25   Water is added to 1000 ml.

GLC analysis

Perkin Elmer Autosystem 9000 Capillary Gas Chromatograph equipped with WCOT
30   fused silica column 12.5 m x 0.25 mm ID x 0.1 μ film thickness 5% phenyl-methyl-
silicone (CP Sil 8 CB from Chrompack).

Carrier gas: Helium.

Injector. PSSI cold split injection (initial temp 50°C heated to 385°C), volume 1.0µl

Detector FID: 395°C

Oven program:	1	2	3
Oven temperature, °C.	90	280	350
Isothermal, time, min.	1	0	10
Temperature rate, °C/min.	15	4	

5

Sample preparation: 30 mg of sample was dissolved in 9 ml Heptane:Pyridin, 2:1 containing internal standard heptadecane, 0.5 mg/ml. 300µl sample solution was

10   transferred to a crimp vial, 300 µl MSTFA (N-Methyl-N-trimethylsilyl-trifluoraceamid) was added and reacted for 20 minutes at 60°C.

Calculation: Response factors for mono-di-triglycerides and free fatty acid are determined from Standard 2 (mono-di-triglyceride). The response factors for Cholesterol, Cholesteryl Palmitate and Cholesteryl Stearate were determined from

15   pure reference materials.

Results: Transferase assay based on phosphatidylcholine and cholesterol as substrate.

In the following the transferase activity of the transferase was tested in a substrate

20   based on phosphatidylcholine and cholesterol according to the following procedure.

450 mg phosphatidylcholine (>95% PC Avanti item no. 441601) and 50 mg cholesterol was dissolved in chloroform and evaporated to dryness under vacuum. 300 mg cholesterol/phosphatidylcholine mixture was transferred to a Wheaton glass and 15

25   ml 50mM HEPES buffer pH 7 was added. The lipid was dispersed in the buffer during agitation.

The substrate was heated to 35 °C during mixing with a magnetic stirrer and 0.25 ml enzyme solution was added. This is a very high water environment of approximately 95% water.

30

Samples of 2 ml were taken out after 0, 5, 10, 15, 25, 40 and 60 minutes reaction time.

Immediately 25 μl 4M HCl was added to acidify the free fatty acid and stop the enzyme reaction. 3.00 ml chloroform was added, and the sample was shaken vigorously on a Whirley for 30 seconds. The sample was centrifuged and 2 ml of the chloroform phase was isolated and filtered through 0.45-μm filters into a 10 ml tared

5   Dram glass. The chloroform was evaporated under a stream of nitrogen at 60°C, and the samples were scaled again. The extracted lipid was analysed by GLC.

The results from the GLC analysis are shown in Table 17. The results are expressed in

10   % calculated on extracted lipid. The amount of fatty acid and cholesterol ester formed as a function of time is illustrated in. Figure 57 It can be concluded from Figure 57 that the enzyme reaction is not linear as a function of time, because an initially strong both hydrolytic and transferase activity is observed. After approximately 10 minutes and until approximately 60 minutes the reaction shows an almost linear response of

15   fatty acid and cholesterol ester formation as a function of time. It was therefore decided to look at the enzymatic reaction in this time interval.

Table 17

Minutes	0	5	10	15	25	40	60
Cholesterol, %	10.064	8.943	8.577	8.656	8.102	7.856	7.809
Cholesterol ester, %	0.000	1.571	2.030	2.058	2.282	2.659	3.081
FFA total, %	0.260	1.197	1.239	1.466	2.445	2.943	3.940

20

From the knowledge about the amount of lipid in the reaction mixture and the amount of enzyme added it was possible to calculate the formation of fatty acid and cholesterol ester expressed in μmol/ml enzyme (Table 18 and Figure 58)

Table 18

Minutes	10	15	25	40	60
	μmol/ml	μmol/ml	μmol/ml	μmol/ml	μmol/ml
FFA total	58.1	68.7	114.6	138.0	184.7
Cholesterol ester	88.8	90.0	99.3	115.6	133.8

From the results in Table 18 and the slope of the curves in Figure 58 it was possible to calculate the amount of fatty acid and cholesterol ester as a function of time expressed

5 in μmol/min per ml enzyme:

The calculation of the hydrolytic activity and the transferase activity is shown in Table 19. The relative transferase activity was determined using the protocol for the determination of % acyltransferase activity as described hereinbefore.

10

Table 19

Hydrolytic activity (fatty acid)	2.52	μmol/min per ml enzyme
Transferase activity(cholesterol ester)	0.94	μmol/min per ml enzyme
Total activity	3.45	μmol/min per ml enzyme
Relative Transferase activity	27.1	%
Relative hydrolytic activity	72.9	%

Screening of other enzymes for transferase activity.

15

The method mentioned above was used to screen different lipolytic enzymes for transferase and hydrolytic activity. The enzymes were tested as shown in Table 20

Table 20

		1	2	3	4	5
Substrate	ml	15	15	15	15	15
#178-9Transferase A. salmonicida 32 PLU-7/ml	ml	0.25				
5% #3016, LIPOPAN® F (F. oxysporum)	ml		0.25			
5%, Thermomyces lanuginosus	ml			0.25		
5% Candida rugosa #2983	ml				0.25	
5% Candida cylindracea #3076	ml					0.25

The substrate containing 300mg phosphatidylcholine/cholesterol dispersed in 50 mM
HEPES buffer pH 7.0 was heated to 35 °C with agitation. Enzyme solution was added
and the sample was kept at 35 °C with agitation. Samples were taken out with regular
interval and extracted with Chloroform. The isolated lipids were analysed by GLC
with results shown in Table 21.

Table 21

Sample								
1	Transferase 178-9							
	Minutes	0	5	10	15	25	40	60
	FFA	1.216	2.516	2.983	2.62	2.894	3.448	3.911
	Cholesterol	7.547	6.438	6.365	6.15	6.136	5.936	5.662
	Chl. Ester	0	1.835	2.177	2.44	2.58	2.851	3.331
2	Fusarium oxysporum (LIPOPAN® F)	0	5	10	15	25	40	60
	FFA	1.216	1.345	1.796	1.95	2.487	2.424	2.977
	Cholesterol	7.547	7.309	7.366	7.33	7.429	7.341	7.326
	Chl. Ester	0	0.26	0.386	0.35	0.267	0.36	0.394

3	Thermomyces lanuginosus	0	5	10	15	25	40	60
	FFA	1.216	0.853	0.875	1	0.896	1.105	1,009
	Cholesterol	7.547	7.384	7.639	7.63	7.675	7.603	7.529
	Chl. Ester	0	0	0	0	0	0	0
4	Candida rugosa (#2938)	0	5	10	15	25	40	60
	FFA	1.216	0.982	0.987	1.02	1.135	1.131	1.15
	Cholesterol	7.547	7.438	7.656	7.66	7.638	7.575	7.585
	Chl. Ester	0	0	0	0	0	0	0
5	Candida cylandracea (#3076)	0	5	10	15	25	40	60
	FFA	1.216	1.032	1.097	1.07	1.203	1.131	1.43
	Cholesterol	7.547	7.502	7.425	7.65	7.619	7.502	7.411
	Chl. Ester	0	0	0	0	0	0	0

From the GLC analysis it was observed that only the lipid acyltransferase (178-9) produced significant amount of cholesterol ester and fatty acids. Phospholipase from Fusarium oxysporum also gave a steady increase in free fatty acid but only an initial small amount formation of cholesterol ester was formed but no increase in cholesterol ester as a function of time was observed.

Based on the knowledge about the amount of lipid substrate and the GLC analyses it was possible to calculate the relative transferase activity and the relative hydrolytic activity based on the results from 10 to 60 minutes reaction time. The results from Transferase 178-9 and Fusarium oxysporum lipase are shown in Table 21. The other enzymes tested showed no activity.

Table 21

	Transferase 178-9	Fusarium oxysporum
Hydrolytic activity, micromole/min per ml enzyme	1.03	0.96
Transferase activity, micromole/min per ml enzyme	0.40	0.01
Total activity, micromole/min per ml enzyme	1.43	0.98
Relative hydrolytic activity	71.8	98.7
Relative transferase activity	28.2	1.3

The result shown in Table 21 confirm a significant transferase activity from the lipid acyltransferase (sample 178-9). It is also observed that the relative transferase activity

5 is in good agreement with the experiment mentioned in Table 19

A very low transferase activity form Fusarium oxysporum phospholipase is however observed. This transferase level is so low that it falls within the uncertainty of the analysis. As expected *Fusarium oxysporum* phospholipase has a significant hydrolytic

10 activity.

Conclusion.

Instead of egg yolk (shown in Example 11) an artificial substrate based on purified

15 phosphatidylcholine and cholesterol was used as a substrate to measure the activity of transferase from *Aeromonas salmonicida*. Between 10 minutes and 60 minutes reaction time the assay gave an almost linear formation of free fatty acids and cholesterol ester as a function of time. Based on the activity between 10 and 60 minutes reaction time the hydrolytic activity and the transferase activity was

20 calculated.

The concentration of substrates in this assay was relatively lower than in egg yolk, and the amount of water in the assay was relatively higher.

Based on the results from the assay of the lipid acyltransferase (in this instance a GCAT) from *Aeromonas salmonicida* in a artificial substrate of phosphatidylcholine/cholesterol in buffer it is concluded that this enzyme has very good transferase activity also in a system with a very high water content.

5 Both assays based on egg yolk (see Example 11) and phosphatidylcholine/cholesterol in buffer (Example 12), can be used to measure the transferase and hydrolytic activity of enzymes. The egg yolk is preferred from the point of view that the hydrolytic and the transferase activity is linear as a function of time, but the phosphatidylcholine/cholesterol in buffer is only linear within a certain time limit.

10

EXAMPLE 13: Food Emulsions

The effect of enzyme modified liquid egg yolk was tested in a standard Food emulsion
15 recipe with 60 % oil.

Standard methods and materials are as per those detailed in the Examples above.

The egg yolk was treated with a lipid acyl transferase from *Aeromonas salmonicida*(
20 #138) or phospholipase, namely a commercially available enzyme LipopanF®
(Novozymes A/S, Denmark) (#2938) as shown in Table 22.

Table 22. Enzyme treatment of egg yolk.

		1	2	3	4
Egg Yolk, Sanofo product no 1123P2	Gram	10	10	10	10
#138, 10 PLU/ml	Ml	1	1		
#2938, 200 PLU/ml	Ml			1	
Water	Ml				1
Reaction time	Minutes	210	360	210	210

25

NZAS-0212658

TLC analysis of the egg yolk lipids from enzyme treated egg yolk (Table 9) is shown in Figures 59 and 60.

In this experiment the dosage of #2938 was increased by a factor of 10 and this gave a
5   very clear activity on egg yolk. The amount of free fatty acid increased significantly and lecithin (PC) was hydrolysed to lysolecithin (LPC). The transferase #138 gave a clear transferase reaction because free cholesterol was converted to cholesterol ester and part of the lecithin was converted to lysolecithin.

10   Another interesting aspect of the enzyme modification was the consistency of the product. The sample treated with Phospholipase #2938 became very solid, whereas the samples treated with the lipid acyltransferase #138 kept the same liquid consistency as the control sample (see Figure 61).

15   These modified egg yolks were tested in a Food Emulsion recipe shown in Table 23.

Table 23. Mayonnaise with enzyme modified egg yolk.

	0	1a	2a	3a	4a
	%	%	%	%	%
Rapsolie	60	60	60	60	60
Egg yolk, Sanofo product no.1123P2	2,8				
Enz. Modified egg yolk no. 1		2,8			
Enz. Modified egg yolk no. 2			2,8		
Enz. Modified egg yolk no. 3				2,8	
Control (untreated) egg yolk no. 4					2,8
Water	39	36,2	36,2	36,2	36,2
Vinegar, 10% acetic acid	1	1	1	1	1

Modified egg yolks 1 and 2 were treated with the lipid acyl transferase; and modified
20   egg yolk 3 was treated with the commercially available phospholipase.

The food emulsion was produced as an oil in water emulsion according to the following procedure: Egg yolk and water was scaled in a beaker. The oil was scaled separately.

5   A Turrax mixer(20000 rpm) was immersed in the water phase. Oil was pumped to the water phase at a constant speed over 2 minutes. The mixing continued for further 1 minute. The vinegar was then added and mixed for 5 seconds.

The stability of the emulsion was tested in a heating cabinet at 100 °C. After 2 hours at 10   100 °C the emulsion was evaluated (see Figure 62).

The emulsion stability of untreated egg yolk was quite good in this experiment. Treatment of egg yolk with the lipid acyltransferase #138 however improved the stability because the amount of water separation was reduced. Egg yolk treated with 15   phospholipase #2938 gave a very unstable emulsion with almost complete separation of the oil and the water phase at 100 °C.

It is considered that in some applications the use of the compositions and methods of the invention can provide enhanced thermal stability of emulsions, such as oil in water 20   salad dressings and the like. This is particularly important in food emulsions which are pasturised to ensure long shelf life and/or are heated prior to serving, e.g. in pre-prepared meals for re-heating prior to serving (e.g. microwave meals). Although not wishing to be bound by any particular theory, it is considered that in some applications the accumulation of free fatty acid may be determental to the thermal stability of such 25   emulsions. It should be recognised that the enhanced thermal stability of the food emulsions produced using the methods of the invention, may not be found, or even desirable, in all food applications. It will be apparent to the person skilled in the art in which applications such characteristics are desirable, and the stability of the emulsions can be easily determiend using a simple heat tests, equivalent to, for example 30   pasturisation and or microwave reheating. The inventors have discovered that in a preferable embodiment the food emulsions obtained using the enzymes of the invention have enhanced thermal stability.

EXAMPLE 14: Transferase reaction in plant sterol enriched egg yolk:

Transferase form *Aeromonas salmonicida* was able to catalyse to formation of
5 lysolecithin, monoglyceride and plant sterol esters in egg yolk enriched with plant
sterol and glycerol. The same enzyme was also tested in a low water system
containing palm oil, lecithin, plant sterol and glycerol By TLC and GLC analyses it
was shown that monoglyceride, and plant sterol esters were produced under these
reaction conditions.

10

Introduction:

The transferase from *Aeromonas salmonicida* was tested for transferase activity in
almost water free system of lecithin, fat, plant sterol and glycerol.

15 Materials:

Egg yolk: Pasteurised liquid egg yolk from Danæg Products A/S, DK- 4000 Roskilde
GCAT transferase purification 178-9 , 32 PLU-7/ml(Journal 2254-100)
Soya lecithin. Yolkin from Aarhus United, Denmark.
Palm oil 43, from Aarhus United, Denmark.
20 L-α Phosphatidylcholine 95% Plant (Avanti #441601)
Sitosterol, Sigma no S5753
Plant Sterol: Generol N122 from Cognis, Germany
Glycerol Item no.085915

25 Results

Initial screening of transferase activity on plant sterol and glycerol was conducted in
egg yolk as shown in Table 24.

30

Table 24

		1	2	3	4
Egg yolk	Gram	1	1	1	1
Glycerol	Gram	0.1	0.1		
Sitosterol:olie 3:7	Gram			0.13	0.13
Transferase #178-9	Units	1		1	
Water			*	.	*

*Water corresponding to the amount of water in the enzyme solution= 83µl

The ingredients were mixed and heated to 37 °C and kept at this temperature during agitation with a magnetic stirrer.

5   0.1 gram samples were taken out after 3 and 23 hours and analysed by TLC.

The results from the TLC analysis is shown in Figure 63.

The result in Figure 63 indicated that both cholesterol and plant sterols were esterified

10   by the transferase reaction, concomitant with the formation of lysolecithin (sample 3 and 4), because almost all free sterol and cholesterol was converted to the corresponding ester in sample 3.

The results also indicated that the sample with only glycerol and egg yolk produced

15   monoglyceride. The amount of monoglyceride needs to be confirmed by GLC analysis. When sterol was added together with glycerol (sample 3) the amount of monoglyceride was very low and not detectable by TLC. This indicated that as long as there were surplus of sterol or cholesterol the transferase reaction using glycerol was modest.

20

In another experiment the transferase enzyme 178-9 was added to a mixture soybean lecithin, glycerol and plant sterol, in order to study the catalytic activity of the enzyme in this reaction mixture.

The composition of the reaction mixtures in these experiments are shown in Table 25

Table 25

		1	2	3	4	5	6
Soya lecithin	gram	1.875	2.25	1.875	2.5	3.5	3.5
Plantesterol; Generol N 122	gram	0.225	0.225	0	0	0.225	0.5
Palm oil 43	gram	2.675	2.25	2.8	2.125	1.062	0.831
Glycerol	gram	0.225	0.275	0.325	0.375	0.248	0.238
Transferase #178 -9, 32 PLU/ml	ml	0.2	0.2	0.2	0.2	0.2	0.2

The experiment was conducted by mixing the lipid components during agitation at 46 °C. The enzyme was added and samples were taken out after 4 and 24 hours.

The samples were analysed by TLC as shown in Figure 64.

Sample from experiment 2, 4 and 5 after 24 hours reaction time were also analysed by GLC with results shown in Table 26

Table 26.

		2	4	5
Glycerol	%	3.16	5.71	4.17
Fatty acids	%	4.23	5.36	6.67
Mono	%	2.24	3.87	3.92
Sterol	%	2.13		2.62
Sterolester	%	2.89		2.14

The results confirmed that transferase 178-9 was able to catalyse to formation plant sterol esters and monoglyceride from a reaction mixture containing soybean lecithin, glycerol and plant sterol. Such reaction mixture could be of interest for use in margarine production where monoglyceride is wanted for their emulsification

5   properties and plant sterol esters for their cholesterol lowering effect.

Conclusion

CGAT transferase from Aeromonas salmonicida was able to catalyse the formation of plant sterol esters and monoglyceride in egg yolk where plant sterol and glycerol was

10   added. The same enzyme also catalysed the formation of plant sterol esters and monoglyceride in a mixture of palm oil, lecithin, plant sterol and glycerol. This enzyme therefore is of interest for use in margarine and other oil containing food products where monoglyceride and lysolecithin are needed for improved emulsification and the plant sterol ester for their cholesterol lowering effects.

15

EXAMPLE 15 : Immobilisation of a lipid acyltransferase from *Aeromonas salmonicida* and the use in the synthesis of Sterol esters.

20

A lipid acyltransferase (in this instance a GCAT) from *A. salmonicida* was immobilised on Celite by acetone precipitation. 10 ml enzyme solution in 20 mM TEA buffer pH 7 was agitated slowly with 0,1 gram Celite 535 535 (from Fluka) for 2 hours at room temperature.

25   50ml cool acetone was added during continued agitation.

The precipitate was isolated by centrifugation 5000 g for 1 minute.

The precipitate was washed 2 times with 20 ml cold acetone.

The Celite was tried at ambient temperature for about 1 hour

30   The immobilised transferase was tested in a oil mixture containing 13 % Phosphatidylcholin and 7 % plant sterol.(Table 27)

Table 27

	%
Avanti lecithin	12.0
Plant sterol, Generol 122N	6.6
Palm 43	71.4
Glycerol	5.0
Immobilised Transferase #178, 45 U/g	2.0
Water	3.0

Lecithin, plant sterol and soybean oil was heated to 46 °C and the plant sterol was dissolved. The immobilised transferase was added.

5   The transferase reaction continued at 46 °C during gentle agitation with a magnetic stirrer. Samples were taken out for analyses after ½, 1 3 6 and 24 hours and analysed by TLC. The reaction was stopped after 24 hours reaction time and the immobilised enzyme was filtered off.

10   The samples were analysed by TLC as shown in Figure 65.

The TLC analysis clearly shows the effect of immobilised transferase from *A. salmonicida* in the transformation of cholesterol into cholesterol ester. It is also observed that small amount of monoglyceride is formed. The enzyme has also been

15   shown to have a high activity in environments with high water content (6- 89 %)water environments, the use of the transferase, and other transferases for use in the invention can therefore also be used in immobilised enzyme applications with a significant water content. This allows the replacement of the solvents used by the current immobilised lipases in the bioconvertion of lipids using transferases.

20

EXAMPLE 16 The Aeromonas hydrophilia transferase can transfer from a phospholipid to a sterol to form a sterol ester, and/or a sugar molecule to form a sugar ester.

25

A lipid acyltransferase from Aeromonas hydrophila expressed in E. coli (Hydro 0303 HVP), labelled #139 was purified on a Chelating Sepharose FF, HR 2.5/10column and analysed for Phospholipase activity. The transferase activity was evaluated in egg
5 yolk for enzyme activity and functionality in egg yolk. The enzyme was also tested in egg yolk containing glucose.

Phospholipase activity.
10 Transferase #139 isolated from a Chelating Sepharose FF, HR 2.5/10column   was assayed by NEFA-PLU(pH7) The activity was 1,15 Units NEFA-PLU/ml.

Egg yolk
In an initial application test transferase #139 was tested in egg yolk according to the
15 · following procedure.
1-gram fresh egg yolk was scaled in a 10 ml flask with screw lid. The enzyme preparation was added and mixed on a Vortex mixer. The sample was placed at 37 °C and agitated with a magnetic stirrer.
The reaction was stopped by adding 7.5 ml Chloroform:Methanol (2:1) and mixed on a
20 Whirley mixer for 30 seconds. The chloroform phase was isolated by centrifugation and 2 µl of the chloroform phase was transferred to a pre-activated silica TLC plate and eluted with running buffer nr. I and another TLC-plate in running buffer IV,

The experimental set up is shown in table 28

25

Table 28

Test	Reaction time	Egg yolk	Transferase #139
no.	min.	gram	units
1	10 .	1	
2	10	1	0.75 NEFA-PLU
3	60	1	0.75 NEFA-PLU

4	300	1	0.75 NEFA-PLU
5	1200	1	
6	1200	1	0.75 NEFA-PLU

TLC analysis are shown in Figure 66 and Figure 67. The TLC analysis clearly demonstrates the transferase reaction of transferase #139. The cholesterol is converted to cholesterol ester and the amount of lecithin is reduced. The results however also indicate that lysolecithin are only accumulated in very small amount because transferase #139 also is active on lysolecithin. This observation is supported by the formation of free fatty acids(FFA).

Egg yolk and glucose

It was earlier shown that a transferase from *Aeromonas salmonicida* (#138) was able to use glucose as acceptor molecule in a transferase reaction. It has also been tested if transferase #139 can use glucose as acceptor molecule. The experimental set up is seen in Table 29.

Table 29

Test	Reaction time	Egg yolk	Glucose, 70%	Transferase #139
no.	Minutes	gram	mg	units
1	10	1	500	
2	10	1	500	1 NEFA-PLU
3	60	1	500	1 NEFA-PLU
4	180	1	500	1 NEFA-PLU
5	300	1	500	1 NEFA-PLU
6	1200	1	500	1 NEFA-PLU
7	1200	1	500	

The reaction products were analysed by TLC (Figures 68 and Figure 69).

The TLC analysis indicates formation of glucose ester after 220 min. reaction time (Figure 69 lane 6) but after 1200 min reaction time no glucose ester is seen.

It must therefore be concluded that transferase #139 has both transferase and
5   hydrolytic activity. This is also supported by the fact that the amount of free fatty acids steadily increases as a function of reaction time.

Resume:

Transferase from *Aeromonas hydrophila* was tested in egg yolk. The results confirm that this enzyme catalyses the formation of cholesterol ester concomitant with the
10   formation of lysolecithin. After extended reaction time when most of the cholesterol is consumed free fatty acid are also formed. It can therefore be concluded that the enzyme has primary transferase activity but also hydrolytic activity was observed when only water was available as donor molecule.

15   In an experiment with egg yolk and glucose it has been observed that transferase from *Aeromonas hydrophila* is able to catalyse the formation of glucose ester *in situ* in a high water food environment (Figure 70).

20   **EXAMPLE 17: Variants of a lipid acyltransferase from *Aeromonas hydrophila* (Ahyd2) (SEQ ID No. 36 (see Figure 71))**

Mutations were introduced using the QuikChange® Multi-Site Directed Mutagenesis kit from Stratagene, La Jolla, CA 92037, USA following the instructions provided by Stratagene.
25

Variants at Tyr256 showed an increased activity towards phospholipids.

Variants at Tyr256 and Tyr260 showed an increased activity towards galactolipids.

30   Variants at Tyr265 show an increased transferase activity with galactolipids as the acyl donor.

The numbers indicate positions on the following sequence: An enzyme from *Aeromonas hydrophila* the amino acid sequence of which is shown as SEQ ID No. 36 in Figure 71 (the underlined amino acids show a xylanase signal peptide). The nucleotide sequence is as shown as SEQ ID No 54 in FIGURE 72.

5

EXAMPLE 18: Use of Acyl-transferase reaction for the production of plant sterol ester and monoglyceride for margarine production.

An acyltransferase from *Aeromonas salmonicida* expressed in *Bacillus subtilis* was

10   tested in a palm oil mixture containing plant lecithin, plant sterol and glycerol. The acyl-transferase showed the ability to utilise both plant sterol and glycerol as acceptor molecules during production of plant sterol ester and monoglyceride. The reaction mixture was used to produce table margarine of good quality based on the monoglyceride in the reaction mixture and at the same time the margarine was

15   enriched with plant sterol ester, which has been shown to have a cholesterol lowering effect.

The aim of this work was to study to possibility to produce monoglyceride and plant sterol ester by enzymatic reaction of lecithin, plant sterol and glycerol dissolved in

20   vegetable fat.

Initial experiments has shown that it was possible to use acyl-transferase from *Aeromonas salmonicida* to produce monoglyceride and plant sterol ester from lecithin, glycerol and plant sterol.

25

In this experiment such reaction mixture was used to produce table margarine.

Materials:

Lipid acyltransferase from *Aeromonas salmonicida*, # 196 C101, 18.6 PLU/g (Journal

30   2254-104)

Palm Oil 43, from Aarhus United, DK

L-α Phosphatidylcholine 95% Plant (Avanti #441601)

Plant Sterol: Generol N122 from Cognis, Germany

Glycerol Item no.085915

Distilled Monoglyceride, Dimodan HP from Danisco.

5   Margarine production.

1.   Blend the water phase ingredients. (If required, pasteurise the water phase by heating to approx. 80°C). Adjust pH 5.5.

10   2.   Melt the fat phase, and temper to approx. 40-45°C..

3.   Heat the emulsifier with some of the oil in a ratio of 1 part emulsifier to 5 parts oil to a temperature (75-80°), which is 5-10°C higher than the melting point of the emulsifier. When this blend is fully melted and

15   well stirred, add it to the remaining heated oil, stirring continuously.

4.   Add the flavouring.

5.   Add the water phase to the fat phase, stirring continuously.

20

6.   Cool in a tube chiller (normal capacity, normal cooling) to an outlet temperature of 8-10°C.

Results

25

Acyltransferase from *A. salmonicida* was tested in an palm oil mixture as shown in Table 30.   Lecithin, plant sterol, glycerol and palm oil was heated to 60°C during agitation in order to solubilize plant sterol and lecithin.

30   Table 30

Substrate: %

Avanti lecithin	12
Plant sterol, Generol 122N	6.6
Palm oil, melting point 43	76.4
Glycerol	5

The substrate was cooled to 48 °C and acyl-transferase #196 was added in the amount shown in Table 31. The reaction mixture was kept at 48 °C for 24 hours during slow agitation.

5

Table 31

	gram
Substrate	220
Transferase # 196 C101,	18.6
PLU/g	15

10 Samples from the reaction mixture were taken out after 1, 4 and 24 hours reaction time, and analysed by TLC in solvent I (Figure 73). The TLC results clearly show the formation of plant sterol ester and monoglyceride. In Figure 73, the first lane is after 1 hour reaction time, Lane 2 is 4 hours reaction time, Lane 3 is 24 hours reaction time and Lane 4 is a plant sterol.

15 The reaction was stopped after 24 hours reaction time and residues of undissolved plant sterol was removed, and the clear solution was used to produce margarine.

Margarine.

The reaction mixture containing monoglyceride and plant sterol ester was used to 20 produce table margarine according to the recipe shown in Table 32.

Table 32

Jour. No 3734	1	2
Water phase		
Water phase	16	16
Salt	0.5	0.5
Skim milk powder	1	1
Potassium sorbate	0.1	0.1
EDTA	0.015	0.015
PH	5.5	5.5
Water phase total	16.6	16.6
Fat phase		
Palm 43	25	25
Rapeseed Oil	75	75
Fat phase total	83.2	78.4
Dimodan HP	0.2	
Reaction mixture		5

The margarine produced from the reaction mixture was evaluated of good quality with
5 good spreadability, and good mouth feel and without any off flavour. The margarine
was compared to be on quality level with the reference margarine produced by using
distilled monoglyceride Dimodan HP.

The only difference observed was that the margarine jour. 3734 no 2 with the reaction
10 mixture was slightly more firm, which was explained by the fact that this recipe
contained more Palm 43 than the reference margarine.

EXAMPLE 19: Use of a lipid acyltransferase during bread production.

One of the limitations of using lipases in bread making is that free fatty acid is formed during the lipase reaction. It is well known that formation of too much free fatty acid

5 will have a negative impact on the baking performance of flour, because the gluten gets too stiff and a bucky (i.e. less elastic) dough is formed which can not expand during fermentation and baking.

Formation of free fatty acid should also be avoided from the point of oxidative

10 stability, because free fatty acids are more prone to lipid oxidation than the corresponding triglyceride.

In the present invention the problems with free fatty acid formation when adding a lipolytic enzyme to a dough has been overcome by using a lipid acyltransferase which,

15 instead of producing free fatty acids, transfers one or more fatty acids from the lipid acyl donor to a non water acceptor molecule present in the dough, such as a carbohydrate, a protein or peptide, or if used in bread with milk fat, a sterol, alternatively or in combinantion other acceptors listed above mat be added to a dough, for example phytosterols or phytostanols. Preferably, the acceptor molecule in a dough

20 may be one or more of glucose, sucrose or maltose and/or other carbohydrates normally available in a dough.

In the following experiments acyl transferase is tested in mini scale baking experiments. The formation of reaction products, and the lipid components in fully

25 proved dough is extracted by water saturated butanol and analysed by HPLC and GLC analysis.

Materials and methods

30 Enzymes:
Acyl Transferase, 550 PLU-7/ml

Lipopan™ F BG, a commercial lipase from Novozymes. 12000 LIPU/g or Grindamyl Exel 16. 12000 LIPU/g

Lecithin powder, 95% phospholipid (available from Danisco A/S Denmark)

Digalactosyldiglyceride from whole wheat flour (from Sigma D4651)

5

Flour : Sølvmel nr. 2001084 (Danish wheat flour, obtained from Havnemølleme, Odense, Denmark)

Mini baking test.

10 Flour, 50 gram, Dry yeast 10 gram, glucose 0.8 gram, salt 0.8 gram, 70 ppm ascorbic acid and, water 400 Brabender units was kneaded in a 50 g Brabender mixing bowl for 5 min at 30 °C.

Resting time was 10 min. at 34 °C . The dough was scaled 15 gram per dough. Then moulded on a special device where the dough is rolled between a wooden plate and a

15 plexiglas frame. The doughs were proofed in tins for 45 min. at 34 °C, and baked in a Voss household oven 8 min. 225 °C.

After baking the breads are cooled to ambient temperature and after 20 min. the breads are scaled and the volume is determined by rape seed displacement method. The breads are also cut and crumb and crust evaluated.

20

Results and conclusion:

Preliminary results indicate that the lipid acyltransferase clearly demonstrates a positive effect on both bread volume and bread appearance. In particular, preliminary

25 results indicate that the use of the lipid acyltransferase results in increased specific bread volume as compared with that obtained with the control (no enzyme) and that obtained with the use of a commercially available lipolytic enzyme, namely Grindamyl Exel 16 or LipopanF™ .

30

EXAMPLE 20: Standard ice cream with dairy fat

The function of emulsifiers used in ice cream is to bring about controlled fat crystallisation and mild destabilization due to protein desorbtion during ageing of the

5   ice cream. This change improves the ice cream quality. Mono-diglycerides are normally used for the production of ice cream, but is also known to use polar emulsifiers like polysorbate and sugar esters in ice cream production in combination with mono-diglyceride to facilitate controlled fat destabilization and produce ice cream with very good creamy and smooth eating texture.

10

Emulsifiers used for ice cream are normally added the ice cream mix as a powder. Recently it has however been shown that mono-diglyceride can bee prodced by enzymatic reaction of the fat in the ice cream recipe using lipases. The problem by using lipases is however that lipases also catalyse the formation of free fatty acids,

15   when water is available in the reaction mixture.

It has however surprisingly been shown that lipid acyl-transferase overcomes the limitation by lipase because acyl-transferase is able to transfer fatty acid from lecithin and other lipids to acceptor molecules like sterol, cholesterol, glucose, glycerol and

20   proteins/peptides without formation of significant amount of free fatty acids.

One of the main ingredients in ice cream is dairy cream containing 38 % milk fat. Dairy cream also contains smaller amount of lecithin, which is a donor molecule for acyl-transferase. (*"Complex milk lipids* account for about 1 % of the total milk fat and

25   are mainly composed of phospholipids". Ref. Ullmann's Encyclopedia of Industrial ChemistryCopyright © 2003 by Wiley-VCH Verlag GmbH & Co. KGaA.). Dairy cream also contains small amount of cholesterol, which is an acceptor molecule for acyl-transferase.

30   From the constituents of ice cream it is thus possible to produce both monoglyceride and polar emulsifiers like lyso-lecithin and sugar ester, which are known for the beneficial effects in ice cream production.

A further beneficial effect form the reaction of acyl-transferase in dairy cream is the formation of cholesterol ester, which might slow down the absorption of cholesterol in the intestine.

5

Ice cream Recipe

	With emulsifier	With enzyme
Dairy cream, 38%	23,65	23,65
Skimmed milk	53,30	53,30
Skimmed milk powder	4,90	11,30
Sugar	12,00	12,00
Glucose sirup, DE 42, 75% TS	4,25	4,25
Glycerol	1,0	1,0
Stabilizer blend	0,2	0,2
Cremodan SE 30	0,6	
Lipid acyl transferase, 500 PLU/g		0,1
Grindsted Flavouring 2976	0,1	0,1
Colour	+	+

Ice Cream production proces.

1. Heat dairy cream, glucose syrup and glycerol to approx. 40 °C Add the lipid acyl
10 transferase and let the mixture react for 30 minutes. A sample is taken out for analysis

2. Heat all the other liquid ingredients to approx. 40 °

3. Add the other dry ingredients. (stabiliser blend is mixed with sugar before addition)

4. When the dry ingredients are dissolved add the dairy cream-glucose mixture.

5. Pasteurise at 80 -85 °C/20-40 seconds

15 **6.** Homogenise at 80 °C (190 bar for recipe 1 and 175 bar for recipe 2)

7. Cool to ageing temperature , 4 °C

8. Freeze in continuous freezer to desired overrun (100% recommended) .

9. Harden in tunnel at -40 °C

10. Store below -25 °C

5

Results:

Uses of Acyl-transferase in the production of ice cream contribute to the production of ice cream with very good taste and excellent creamy mouth feel comparable the ice

10 cream produced by using a commercial emulsifier Cremodan SE 30. The melt down of the ice cream produced by the lipid acyl transferase is also improved.

Example 21: Acyl transferase in Cheese.

15 Cheese is the fresh or matured solid or semisolid product obtained by coagulating milk, skimmed milk, partly skimmed milk, cream, whey cream, or buttermilk, or any combination of these materials, through the action of rennet or other suitable coagulating agents, and partially draining the whey that results from such coagulation.

20

The cheese yield depends primarily on the fat and protein contents of the milk. The salt (particularly calcium salts) and protein concentrations, as well as the acidity, are very important for coagulation. (ref. Ullmann's Encyclopedia of Industrial Chemistry Copyright © 2003 by Wiley-VCH Verlag GmbH & Co).

25

Such effort has been made in order to optimise and increase the cheese yield by optimisation of the cheese making procedure (USP 4,959,229) or by using improved clotting method (USP 4,581,240), which increase the amount of whey protein in the curd.

In the present invention the amount of whey protein in the curd is increased by enzymatic modification of the whey protein · by treatment of the milk during cheese making with a lipid acyl transferase.

5

When a fatty acid is covalently linked to a non-membrane protein like β-lactoglobulin, the physical and functional properties will change drastically.

For cheese production of the present invention acyl transferase is added to the milk before or at the same time as rennet is added to the milk.

10

During casein precipitation acyl transferase is able to use lecithin and other lipids in the milk as doner and peptides or protein as acceptor molecule during formation of acylated protein or acylated peptides.

15

The change in hydrophobic properties of milk protein contributes to increased protein precipitation in the curd during cheese production.

Since the increase in cheese yield obtained by the present invention originates from increased retention in the cheese coagulum of proteins that are normally lost in the whey, a suitable method, directly related to the mechanism of the invention, is based on determination of the amount of protein that ends up in the whey. Less protein in the whey necessarily means more protein in the curd, and higher cheese yield.

20

The test for the amount of protein in the whey can be performed in the following way. Skim or whole milk is warmed to a temperature suitable for rennet coagulation, typically 30-35oC in a 100 ml beaker. Optionally 1% of a bulk lactic acid bacteria starter is added, and standard rennet is added in an amount corresponding to e.g. 0.03-

5 0.05%. When the milk has turned into a coagulum solid enough to allow it to be cut into cubes with a side length of about 0.5 cm, such cutting is performed with a sharp knife. Syneresis is thereby initiated, and after 30 min holding period, that allows the curd to settle, a whey sample is withdrawn, and centrifuged in a laboratory centrifuge for 10 min. This sample is analyzed for protein content, using e.g. the Kjeldahl

10 method. Alternatively, and/or as a supplement, the sample may be analyzed with methods that allow the type and quantity of the individual protein components to be established.

EXAMPLE 22 "Assay in Low Water Environment"

15

Transferase reactions of lipolytic enzymes in low water environment .

Procedure

20 Materials.

Cholesterol Sigma cat. C 8503

L-alpha-Phosphatidylcholine 95% (Plant) Avanti #441601

Soybean oil, Aarhus United, DK.

Chloroform, Analytical grade

25

Enzymes.

#179, GCAT from *A. salmonicida*

#2427, Phospholipase A1 from *Fusarium oxysporum*. LIPOPAN® F from Novozymes, Denmark

30 #1991, Phospholipase A2 from Pancreas, LIPOMOD 22L from Biocatalysts, UK

#2373, *Candida Antarctica* lipase, Novozyme 525 L from Novozymes Denmark.

<u>Enzyme assay</u>

13.1 % Lecithin and 6.6% cholesterol was dissolved in soybean oil by heating to
60 °C during agitation

The substrate was scaled in a 20ml Wheaton glass and heated to 46 °C

5   Water and enzyme solution was added and a stopwatch is started.

At regular intervals 50 mg samples ware transferred to a 10ml Dram glass and frozen.

The isolated lipids were analysed by GLC

<u>GLC analysis</u>

10

Perkin Elmer Autosystem 9000 Capillary Gas Chromatograph equipped with WCOT
fused silica column 12.5 m x 0.25 mm ID x 0.1 μ film thickness 5% phenyl-methyl-
silicone (CP Sil 8 CB from Chrompack).

Carrier gas: Helium.

15   Injector. PSSI cold split injection (initial temp 50°C heated to 385°C), volume 1.0μl

Detector FID: 395°C

Oven program (used since 30.10.2003):	1	2	3
Oven temperature, °C.	90	280	350
Isohtermal, time, min.	1	0	10
20 Temperature rate, °C/min.	15	4	

Sample preparation: 30 mg of sample was dissolved in 9 ml Heptane:Pyridin, 2:1
containing internal standard heptadecane, 0.5 mg/ml. 300μl sample solution was
transferred to a crimp vial, 300 μl MSTFA (N-Methyl-N-trimethylsilyl-
25   trifluoraceamid) was added and reacted for 20 minutes at 60°C.

Calculation: Response factors for mono-di-triglycerides and free fatty acid were
determined from Standard 2 (mono-di-triglyceride), for Cholesterol, Cholesteryl
palmitate and Cholesteryl stearate the response factors were determined from pure
30   reference material (weighing for pure material 10mg).

Results

The experiment was set up as shown in Table 33

The substrate based on soybean oil containing 13.1 % lecithin and 6.6% cholesterol was heated to 46°C. The enzyme solution was added and a stopwatch started.

5 After 30, 60 and 120 minutes reaction time samples were taken out for GLC analysis.

Table 33

		1	2	3	4	5
Substrate	gram	5	5	5	5	5
Transferase #179-C72, 56 PLU-7/ml	ml		0.3			
#2427, 200 PLU-7/ml	ml			0.3		
Pancreas PLA 2 #1991 6300 PLU/ml	ml				0.3	
Novozyme 525 L, #2373, 200 LIPU/ml	ml					0.3
Water	ml	0.3				
% water		6	6	6	6	6

10

The results from the GLC analysis is shown in Table 34 . The results are expressed in percent based total sample composition. Based on the GLC results it was possible to

15 calculate the amount of fatty acid and cholesterol ester produced by enzymatic reaction relative to the control sample without enzyme added. Under these experimental conditions the total enzymatic activity was estimated as the hydrolytic activity measured as free fatty acid formation and the transferase activity estimated as cholesterol ester formation. From these results and the information about molecular

20 weight of fatty acid and cholesterol ester it was possible to calculate to relative molar hydrolytic activity and the relative molar transferase activity as shown in Table 35.

Table 34

Enzyme	Reaction time minutes	Fatty acid %	cholesterol %	Cholesterol ester %
Control	120	0.533	7.094	0.000
#179	30	0.770	5.761	2.229
#179	60	0.852	5.369	2.883
#179	120	0.876	4.900	3.667
#2427	30	3.269	7.094	0.000
#2427	60	3.420	7.094	0.000
#2427	120	3.710	7.094	0.000
#1991	30	2.871	7.094	0.000
#1991	60	3.578	7.094	0.000
#1991	120	3.928	7.094	0.000
#2373	30	1.418	7.094	0.000
#2373	60	1.421	7.094	0.000
#2373	120	1.915	7.094	0.000

5   Table 35

Enzyme	Reaction time minutes	Fatty acid produced	Cholesterol used	Cholesterol ester produced	Hydrolytic activity %	Transferase activity %
#179	30	0.238	1.334	2.229	20	80
#179	60	0.319	1.725	2.883	21	79
#179	120	0.343	2.195	3.667	18	82
#2427	30	2.737	0.000	0.000	100	0

#2427	60	2.887	0.000	0.000	100	0
#2427	120	3.177	0.000	0.000	100	0
#1991	30	2.338	0.000	0.000	100	0
#1991	60	3.046	0.000	0.000	100	0.
#1991	120	3.395	0.000	0.000	100	0
#2373	30	0.885	0.000	0.000	100	0
#2373	60	0.888	0.000	0.000	100	0
#2373	120	1.383	0.000	0.000	100	0

Conclusion

In these experiments it was observed that all the tested enzymes showed hydrolytic activity because the amount of fatty acid increased. However the only enzyme which

5 showed transferase activity was GCAT from *A. salmonicida*. It is therefore concluded that in an oily system with lecithin and cholesterol containing 6% water phospholipase A1 from *Fusarium oxysporum* , phospholipase A2 from *pancreas* and a lipase from *Candida antarctica* only showed hydrolytic activity.

10 All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the present invention will be apparent to those skilled in the art without departing from the scope and spirit of the present invention. Although the present invention has been described in connection with specific preferred embodiments, it should be

15 understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in biochemistry and biotechnology or related fields are intended to be within the scope of the following claims.

20

CLAIMS

1. A method for the *in situ* production of an emulsifier in a foodstuff, wherein the method is such that the emulsifier is produced, wherein the method comprises the step of adding a lipid acyltransferase to the foodstuff.

2. A method according to claim 1 wherein at least 2 emulsifiers are produced.

3. A method according to claim 1 or claim 2 wherein the emulsifier is produced without increasing or substantially increasing the free fatty acids in the foodstuff.

4. A method according to any one of claims 1-3 wherein the lipid acyltransferase is one which is capable of transferrin an acyl group from a lipid to one or more of the following acyl aceptors: a sterol, a stanol, a carbohydrate, a protein or a sub-unit thereof, glycerol.

5. A method according to claim 2 wherein at least one of the emulsifiers is a carbohydrate ester.

6. A method according to claim 2 wherein at least one of the emulsifiers is a protein ester.

7. A method according to any one of the preceding claims wherein one or more of a sterol ester or a stanol ester or a protein ester or a carbohydrate ester or a diglyceride or a monoglyceride is produced *in situ* in the foodstuff.

8. A method according to claim 7 wherein the sterol ester is one or more of alpha-sitosterol ester, beta-sitosterol ester, stigmasterol ester, ergosterol ester, campesterol ester or cholesterol ester.

9. A method according to claim 6 wherein the stanol ester is one or more beta-sitostanol or ss-sitostanol.

10. A method according to any one of the preceding claims wherein the lipid acyltransferase is characterised as an enzyme which possesses acyl transferase activity and which comprises the amino acid sequence motif GDSX, wherein X is one or more of the following amino acid residues L, A, V, I, F, Y, H, Q, T, N, M or S..

11. A method according to any one of the preceding claims wherein the lipid acyltransferase enzyme comprises H-309 or comprises a histidine residue at a position corresponding to His-309 in the amino acid sequence of the *Aeromonas hydrophila* lipolytic enzyme shown as SEQ ID No. 2 or SEQ ID No. 32.

12. A method according to any one of the preceding claims wherein the lipid acyltransferase is obtainable from an organism from one or more of the following genera: *Aeromonas, Streptomyces, Saccharomyces, Lactococcus, Mycobacterium, Streptococcus, Lactobacillus, Desulfitobacterium, Bacillus, Campylobacter, Vibrionaceae, Xylella, Sulfolobus, Aspergillus, Schizosaccharomyces, Listeria, Neisseria, Mesorhizobium, Ralstonia, Xanthomonas* and *Candida*.

13. A method according to any one of the preceding claims wherein the lipid acyltransferase comprises one or more of the following amino acid sequences: (i) the amino acid sequence shown as SEQ ID No. 2; (ii) the amino acid sequence shown as SEQ ID No. 3; (iii) the amino acid sequence shown as SEQ ID No. 4; (iv) the amino acid sequence shown as SED ID No. 5; (v) the amino acid sequence shown as SEQ ID No. 6; (vi) the amino acid sequence shown as SEQ ID No. 12, (vii) the amino acid sequence shown as SEQ ID No. 20, (viii) the amino acid sequence shown as SEQ ID No. 22, (ix) the amino acid sequence shown as SEQ ID No. 24, (x) the amino acid sequence shown as SEQ ID No. 26, (xi) the amino acid sequence shown as SEQ ID No. 28, (xii) the amino acid sequence shown as SEQ ID No. 30, (xiii) the amino acid sequence shown as SEQ ID No. 32, (xiv) the amino acid sequence shown as SEQ ID No. 34, or an amino acid sequence which has 75% or more identity with any one of the sequences shown as SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 12, SEQ ID No. 20, SEQ ID No. 22, SEQ ID No. 24, SEQ ID No. 26, SEQ ID No. 28, SEQ ID No. 30, SEQ ID No. 32 or SEQ ID No. 34.

14. A method according to any one of the preceding claims, wherein the emulsifier is one or more of the following: a monoglyceride, a lysophosphatidylcholine, DGMG.

15. Use of a lipid acyltransferase to prepare from a food material a foodstuff comprising an emulsifier, wherein the emulsifier is produced without increasing or without substantially increasing the free fatty acids in the foodstuff, and wherein the emulsifier is generated from constituents of the food material by the lipid acyltransferase.

16. Use according to claim 15 wherein at least two emulsifiers are produced.

17. Use according to claim 16 wherein at least one of the emulsifiers is a carbohydrate ester.

18. Use according to claim 16 wherein at least one of the emulsifiers is a protein ester.

19. Use according to any one of claims 15-18 wherein one or more of a sterol ester or a stanol ester or a protein ester or a carbohydrate ester or a diglyceride or a monoglyceride is also produced *in situ* in the foodstuff.

20. Use according to claim 19 wherein the sterol ester is one or more of alpha-sitosterol ester, beta-sitosterol ester, stigmasterol ester, ergosterol ester, campesterol ester or cholesterol ester.

21. Use according to claim 20 wherein the stanol ester is one or more beta-sitostanol or ss-sitostanol.

22. Use according to any one of claims 15 to 21 wherein the lipid acyltransferase is characterised as an enzyme which possesses acyl transferase activity and which comprises the amino acid sequence motif GDSX, wherein X is one or more of the following amino acid residues L, A, V, I, F, Y, H, Q, T, N, M or S..

23. Use according to any one of claims 15-22 wherein the lipid acyltransferase enzyme comprises H-309 or comprises a histidine residue at a position corresponding to His-309 in the amino acid sequence of the *Aeromonas hydrophila* lipolytic enzyme shown as SEQ ID No. 2 or SEQ ID No. 32.

24. Use according to any one of claims 15-23 wherein the lipid acyltransferase is obtainable from an organism from one or more of the following genera: *Aeromonas, Streptomyces, Saccharomyces, Lactococcus, Mycobacterium, Streptococcus, Lactobacillus, Desulfitobacterium, Bacillus, Campylobacter, Vibrionaceae, Xylella, Sulfolobus, Aspergillus, Schizosaccharomyces, Listeria, Neisseria, Mesorhizobium, Ralstonia, Xanthomonas* and *Candida*.

25. Use according to any one of claims 15-24 wherein the lipid acyltransferase comprises one or more of the following amino acid sequences: (i) the amino acid sequence shown as SEQ ID No. 2; (ii) the amino acid sequence shown as SEQ ID No. 3; (iii) the amino acid sequence shown as SEQ ID No. 4; (iv) the amino acid sequence shown as SED ID No. 5; (v) the amino acid sequence shown as SEQ ID No. 6; (vi) the amino acid sequence shown as SEQ ID No. 12, (vii) the amino acid sequence shown as SEQ ID No. 20, (viii) the amino acid sequence shown as SEQ ID No. 22, (ix) the amino acid sequence shown as SEQ ID No. 24, (x) the amino acid sequence shown as SEQ ID No. 26, (xi) the amino acid sequence shown as SEQ ID No. 28, (xii) the amino acid sequence shown as SEQ ID No. 30, (xiii) the amino acid sequence shown as SEQ ID No. 32, (xiv) the amino acid sequence shown as SEQ ID No. 34, or an amino acid sequence which has 75% or more identity with any one of the sequences shown as SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 12, SEQ ID No. 20, SEQ ID No. 22, SEQ ID No. 24, SEQ ID No. 26, SEQ ID No. 28, SEQ ID No. 30, SEQ ID No. 32 or SEQ ID No. 34.

26. Use according to any one of claims 15-25, wherein the emulsifier is one or more of the following: a monoglyceride, a lysophosphatidylcholine, DGMG.

27. A food or feed enzyme composition which contains a lipid acyltransferase.

28. A food or feed enzyme composition according to claim 27 wherein the lipid acyltransferase is characterised as an enzyme which possesses acyl transferase activity and which comprises the amino acid sequence motif GDSX, wherein X is one or more of the following amino acid residues L, A, V, I, F, Y, H, Q, T, N, M or S.

29. A food or feed enzyme composition according to claim 27 or claim 28 wherein the lipid acyltransferase enzyme comprises H-309 or comprises a histidine residue at a position corresponding to His-309 in the amino acid sequence of the *Aeromonas hydrophila* lipolytic enzyme shown as SEQ ID No. 2 or SEQ ID No. 32.

30. A food or feed enzyme composition according to any one of claims 27-29 wherein the lipid acyltransferase is obtainable from an organism from one or more of the following genera: *Aeromonas, Streptomyces, Saccharomyces,*

Lactococcus, Mycobacterium, Streptococcus, Lactobacillus, Desulfitobacterium, Bacillus, Campylobacter, Vibrionaceae, Xylella, Sulfolobus, Aspergillus, Schizosaccharomyces, Listeria, Neisseria, Mesorhizobium, Ralstonia, Xanthomonas and *Candida.*

31. A food or feed enzyme composition according to any one of claims 27-30 wherein the lipid acyltransferase comprises one or more of the following amino acid sequences: (i) the amino acid sequence shown as SEQ ID No. 2; (ii) the amino acid sequence shown as SEQ ID No. 3; (iii) the amino acid sequence shown as SEQ ID No. 4; (iv) the amino acid sequence shown as SED ID No. 5; (v) the amino acid sequence shown as SEQ ID No. 6; (vi) the amino acid sequence shown as SEQ ID No. 12, (vii) the amino acid sequence shown as SEQ ID No. 20, (viii) the amino acid sequence shown as SEQ ID No. 22, (ix) the amino acid sequence shown as SEQ ID No. 24, (x) the amino acid sequence shown as SEQ ID No. 26, (xi) the amino acid sequence shown as SEQ ID No. 28, (xii) the amino acid sequence shown as SEQ ID No. 30, (xiii) the amino acid sequence shown as SEQ ID No. 32, (xiv) the amino acid sequence shown as SEQ ID No. 34, or an amino acid sequence which has 75% or more identity with any one of the sequences shown as SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 12, SEQ ID No. 20, SEQ ID No. 22, SEQ ID No. 24, SEQ ID No. 26, SEQ ID No. 28, SEQ ID No. 30, SEQ ID No. 32 or SEQ ID No. 34.

32. Use of a food or feed enzyme composition according to any one of claims 27-31 in accordance with any one of claims 15-26 or in the method according to any one of claims 1-14.

33. A food stuff obtainable by the method according to any one of claims 1-14.

34. An immobilised lipid acyltransferase enzyme.

35. An immobilised lipid acyltransferase according to claim 34 wherein the lipid acyltransferase is characterised as an enzyme which possesses acyl transferase activity and which comprises the amino acid sequence motif GDSX, wherein X is one or more of the following amino acid residues L, A, V, I, F, Y, H, Q, T, N, M or S.

36. An immobilised lipid acyltransferase according to claim 34 or claim 35 wherein the lipid acyltransferase enzyme comprises H-309 or comprises a histidine residue at a position corresponding to His-309 in the amino acid sequence of the *Aeromonas hydrophila* lipolytic enzyme shown as SEQ ID No. 2 or SEQ ID No. 32.

37. An immobilised lipid acyltransferase according to any one of claims 34-36 wherein the lipid acyltransferase is obtainable from an organism from one or more of the following genera: *Aeromonas, Streptomyces, Saccharomyces, Lactococcus, Mycobacterium, Streptococcus, Lactobacillus, Desulfitobacterium, Bacillus, Campylobacter, Vibrionaceae, Xylella, Sulfolobus, Aspergillus, Schizosaccharomyces, Listeria, Neisseria, Mesorhizobium, Ralstonia, Xanthomonas* and *Candida.*

38. An immobilised lipid acyltransferase according to any one of claims 34-37 wherein the lipid acyltransferase comprises one or more of the following amino acid sequences: (i) the amino acid sequence shown as SEQ ID No. 2; (ii) the amino acid sequence shown as SEQ ID No. 3; (iii) the amino acid sequence shown as SEQ ID No. 4; (iv) the amino acid sequence shown as SED ID No. 5; (v) the amino acid sequence shown as SEQ ID No. 6; (vi) the amino acid sequence shown as SEQ ID No. 12, (vii) the amino acid sequence shown as SEQ ID No. 20, (viii) the amino acid sequence shown as SEQ ID No. 22, (ix) the amino acid sequence shown as SEQ ID No. 24, (x) the amino acid sequence shown as SEQ ID No. 26, (xi) the amino acid sequence shown as SEQ ID No. 28, (xii) the amino acid sequence shown as SEQ ID No. 30, (xiii) the amino acid sequence shown as SEQ ID No. 32, (xiv) the amino acid sequence shown as SEQ ID No. 34, or an amino acid sequence which has 75% or more identity with any one of the sequences shown as SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 12, SEQ ID No. 20, SEQ ID No. 22, SEQ ID No. 24, SEQ ID No. 26, SEQ ID No. 28, SEQ ID No. 30, SEQ ID No. 32 or SEQ ID No. 34.

39. A method of identifying a suitable lipid acyltransferase for use in accordance with the present invention, comprising the steps of testing an enzyme of interest using one or more of the "Transferase Assay in a Low Water

environment", the "Transferase Assay in High Water Egg Yolk" or the "Transferase Assay in Buffered Substrate", and selecting a lipid acyltransferase if it is one which has one or more of the following characteristics: (a) when tested using the "Transferase Assay in a Low Water Environment", measured

5 after a time period selected from 30, 20 or 120 minutes, has a relative transferase activity of at least 1%; (b) when tested using the "Transferase Assay in High Water Egg Yolk" in an egg yolk with 54% water, has up to 100% relative transferase activity; or (c) when tested using the "Transferase Assay in Buffered Substrate" has at least 2% acyltransferase activity.

10 40. A method according to claim 39 wherein the lipid acyltransferase is selected if it is one which has more than two of the following characteristics (a) when tested using the "Transferase Assay in a Low Water Environment", measured after a time period selected from 30, 20 or 120 minutes, has a relative transferase activity of at least 1%; (b) when tested using the "Transferase Assay in High

15 Water Egg Yolk" in an egg yolk with 54% water, has up to 100% relative transferase activity; or (c) when tested using the "Transferase Assay in Buffered Substrate" has at least 2% acyltransferase activity.

 41. A method according to claim 39 wherein the lipid acyltransferase is selected if it is one which has more than three of the following characteristics (a) when

20 tested using the "Transferase Assay in a Low Water Environment", measured after a time period selected from 30, 20 or 120 minutes, has a relative transferase activity of at least 1%; (b) when tested using the "Transferase Assay in High Water Egg Yolk" in an egg yolk with 54% water, has up to 100% relative transferase activity; or (c) when tested using the "Transferase

25 Assay in Buffered Substrate" has at least 2% acyltransferase activity.

 42. A method according to claim 39 wherein the lipid acyltransferase is selected if it is one which has all of the following characteristics (a) when tested using the "Transferase Assay in a Low Water Environment", measured after a time period selected from 30, 20 or 120 minutes, has a relative transferase activity of

30 at least 1%; (b) when tested using the "Transferase Assay in High Water Egg Yolk" in an egg yolk with 54% water, has up to 100% relative transferase

activity; or (c) when tested using the "Transferase Assay in Buffered Substrate" has at least 2% acyltransferase activity.

43.   A lipid acyltransferase identified using a method according to any one of claims 39-42.

5

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ABSTRACT

METHOD

A method for the *in situ* production of an emulsifier in a foodstuff, wherein a lipid acyltransferase is added to the foodstuff. Preferably the emulsifier is produced

5 without an increase or without a substantial increase in the free fatty acid content of the foodstuff. Preferably, the lipid acyltransferase is one which is capable of transferring an acyl group from a lipid to one or more of the following acyl aceptors: a sterol, a stanol, a carbohydrate, a protein or a sub-unit thereof, glycerol. Preferably, in addition to an emulsifier one or more of a stanol ester or a stanol ester or a protein

10 ester or a carbohydrate ester or a diglyceride or a monoglyceride may be produced. One or more of these may function as an additional emulsifier.

1

Figure 1

SEQ ID No. 1

```
  1 ivafGDSlTd geayygdsdg ggwgaqladr Ltallrlrar prgvdvfnrg isGrtsdGrl
 61 ivDalvallF laqslglpnL pPYLsgdflr GANFAsagAt llptsgpfli QvqFkdfksq
121 vlelrqalgl lqellrllpv ldakspdlvt imiGtNplit saffgpkste sdrnvsvpef
181 kdnlrqlikr Lrsnngarii vlitlvilnl gplGClPlkl alalassknv dasgclerln
241 eavadfneal relaiskled qlrkdglpdv kgadvpyvDl ysifqdldgi qnpsayvyGF
301 ettkaCCGyG gryNynrvCG naglcnvtak aCnpssylls flfwDgfps ekGykavAea
361 l
```

Figure 2

SEQ ID No. 2

```
  1 mkkwfvcllg lvaltvqaad srpafsrivm fgdslsdtgk myskmrgylp ssppyyegrf
 61 sngpvwleql tnefpgltia neaeggptav aynkiswnpk yqvinnldye vtqflqkdsf
121 kpddlvilwv gandylaygw nteqdakrvr daisdaanrm vlngakeill fnlpdlgqnp
181 sarsqkvvea ashvsayhnq lllnlarqla ptgmvklfei dkqfaemlrd pqnfglsdqr
241 nacyggsyvw kpfasrsast dsqlsafnpq erlaiagnpl laqavaspma arsastlnce
301 gkmfwdqvhp ttvvhaalse paatfiesqy eflah
```

Figure 3

SEQ ID No. 3

```
  1 mkkwfvcllg lialtvqaad trpafsrivm fgdslsdtgk myskmrgylp ssppyyegrf
 61 sngpvwleql tkqfpgltia neaeggatav aynkiswnpk yqvynnldye vtqflqkdsf
121 kpddlvilwv gandylaygw nteqdakrvr daisdaanrm vlngakqill fnlpdlgqnp
181 sarsqkvvea vshvsayhnk lllnlarqla ptgmvklfei dkqfaemlrd pqnfglsdve
241 npcydggyvw kpfatrsvst drqlsafspq erlaiagnpl laqavaspma rrsasplnce
301 gkmfwdqvhp ttvvhaalse raatfietqy eflahg
```

Figure 4

SEQ ID No. 4

```
  1 mpkpalrrvm tatvaavgtl algltdatah aapaqatptl dyvalgdsys agsgvlpvdp
 61 anllclrsta nyphviadtt garltdvtcg aaqtadftra qypgvapqld algtgtdlvt
121 ltiggndmst finaitacgt agvlsggkgs pckdrhgtsf ddeieantyp alkeallgvr
181 arapharvaa lgypwitpat adpscflklp laagdvpylr aiqahlndav rraaeetgat
241 yvdfsgvsdg hdaceapgtr wiepllfghs lvpvhpnalg errmaehtmd vlgld
```

Figure 5

SEQ ID No. 5

```
  1 mpkpalrrvm tatvaavgtl algltdatah aapaqatptl dyvalgdsys agsgvlpvdp
 61 anllclrsta nyphviadtt garltdvtcg aaqtadftra qypgvapqld algtgtdlvt
121 ltiggndnst finaitacgt agvlsggkgs pckdrhgtsf ddeieantyp alkeallgvr
181 arapbarvaa lgypwitpat adpscflklp laagdvpylr aiqahlndav rraaeetgat
241 yvdfsgvsdg hdaceapgtr wiepllfghs lvpvhpnalg errmaehtmd vlgld
```

Figure 6

SEQ ID No. 6

```
  1 mdyekfllfg dsitefafnt rpiedgkdqy algaalvney trkmdilqrg fkgytsrwal
 61 kilpeilkhe snivmatifl gandacsagp qsvplpefid nirqmvslmk syhirpiiig
121 pglvdrekwe kekseeialg yfrtnenfai ysdalaklan eekvpfvaln kafqqeggda
181 wqqlltdglh fsgkgykifh dellkvietf ypqyhpknmq yklkdwrdvl ddgsnims
```

Figure 7

Alignment of pfam00657.6 consensus sequence with P10480

```
            *->ivafGDSlTdg................eayygdsdgggwgagladrL
              iv+fGDSl+d+++  ++ ++  +++++++ +++s+g  w ++l + +
   P10480   28    IVMFGDSLSDTgkmyskmrgylpssppYYEGRFSNGPVWLEQLTNEF 74

            tall..rlrarprgvdvfnrgisGrtsdGrlivDalvallFlaqslglpn
              + 1    + +++++++ +n+  +
   P10480   75 PGLTiaNEAEGGPTAVAYNKISWNPK------------------ 100

            LpPYLsgdflrGANFAsagAtIlptsgpfliQvqFkdfksqvlelrqalg
                                                      ++  ++
   P10480  101 ---------------------------------YQVINN 106

            llqellrllpvldakspdlvtimiGtNDlitsaffgpkstesdrnvsvpe
            l++e+ ++l +++ k+ dlv+++G+ND+      ++ ++ +++++
   P10480  107 LDYEVTQFLQKDSFKPDDLVILWVGANDY-------LAYGWNTEQDAKR 148

            fkdnlrqlikrLrsnngariivlitlvilnlgplGClPlklalalasskn
            ++d +++++r+    nga+      +++nl+ lG+ P+
   P10480  149 VRDAISDAANRMV-LNGAK-----EILLFNLPDLGQNPS---------- 181

            vdasgclerlneavadfnealrelaiskledqlrkdglpdvkgadvpyvD
            ++++ +e +  ++a++n++l +la     +ql+++g+++++++d ++++
   P10480  182 ARSQKVVEAASHVSAYHNQLLLNLA-----RQLAPTGMVKLFEIDKQFAE 226

            lysifqdldgiqnpsayv.y....GFe.ttkaCCGyGgr.yNyn.rv.CG
            +  +q+++ + + +a++++  +++ +++a++++++ +N++r+ ++
   P10480  227 MLRDPQNFGLSDQRNACYgGsyvwKPFaSRSASTDSQLSaFNPQeRLaIA 276

            nag.l.c.nvtakaC.npssyll.sflfwDgfHpsekGykavAeal<-*
            +++ 1 +  ++++a++ +s+ ++++++fwD++Hp+   ++a+ e
   P10480  277 GNPllaQaVASPMAArSASTLNCeGRMFWDQVHPTTVVHAALSEPA 322
```

Alignment of pfam00657.6 consensus sequence with AAG09804

```
            *->ivafGDSlTdg...............eayygdsdgqgwgagladrL
              iv+fGDSl+d+++  ++ ++  +++++++ +++s+g  w ++l + +
 AAG09804   28    IVMFGDSLSDTgkmyskmrgylpssppYYEGRFSNGPVWLEQLTKQF 74

            tallrlrarprgvdvfnrgisGrtsdGrlivDalvallFlaqslglpnLp
              +g+++ n + +G+t
 AAG09804   75 ----------PGLTIANEAEGGAT----------------------- 88

            PYLsgdflrGANFAsagAtIlptsgpfliQvqFkdfksqvlelrqa....
                                          ++++ + ++++ +
 AAG09804   89 -------------------------------AVAYNKISWNpkyq 102

            ..lgllqellrllpvldakspdlvtimiGtNDlitsaffgpkstesdrnv
            ++l++e+ ++l +++ k+ dlv+++G+ND+      ++ ++ ++
 AAG09804  103 vyNNLDYEVTQFLQKDSFKPDDLVILWVGANDY--------LAYGWNTEQ 144

            svpefkdnlrqlikrLrsnngariivlitlvilnlgplGClPlklalala
            ++++++d +++++r+    nga+      +++++nl+ lG+ P+
 AAG09804  145 DAKRVRDAISDAANRMV-LNGAK-----QILLFNLPDLGQNPS------ 181

            ssknvdasgclerlneavadfnealrelaiskledqlrkdglpdvkgadv
                ++++ +e +  ++a++n++l +la     +ql+++g+++++++d
 AAG09804  182 ----ARSQKVVEAVSHVSAYHNKLLLNLA-----RQLAPTGMVKLFEIDK 222

            pyvDlysifqdldgiqnpsayv.y....GFe.ttkaCCGyGgr.yNyn.r
            +++++  +q+++ + ++ +++++  ++ t++ +++ +++ + +++r
 AAG09804  223 QFAEMLRDPQNFGLSDVENPCYdGgyvwKPFaTRSVSTDRQLSaFSPQeR 272

            v.CGnag.l.c.nvtakaC.npssyll.sflfwDgfHpsekGykavAeal
            +  +++++ 1 +  +++a++ +s ++++++fwD++Hp+   ++a+ e+
 AAG09804  273 LaIAGNPllaQaVASPMARrSASPLNCeGRMFWDQVHPTTVVHAALSERA 322

            <-*
```

AAG09804 — — —

Alignment of pfam00657.6 consensus sequence with NP_631558

```
                    *->ivafGDSlTdgeayygdsdgggwgagladrLtallrlrarprgvdvf
                       +va+GDS ++g        +g + +++L      + + + ++  +
NP_631558    42        YVALGDSYSAG---------SGVLPVDPANL----LCLRSTANYPHV 75

                       nrgisGrtsdGrlivD.a.l.vallFlaqslglpnLpPYLsgdflrGANF
                       + ++G++        D + + +
NP_631558    76        IADTTGAR-----LTDvTcGaAQ------------------------- 93

                       AsagAtIlptsgpfliQvqFkdfksqvlelrqalgllqellrllpvldak
                                              +++      ++ +  ++ +++
NP_631558    94        -----------------------TADFTRAQYPGVAPQLDALGT 114

                       spdlvtimiGtNDl.................itsaffgpkstesdrnvsvp
                       + dlvt+ iG+ND ++  +  +  ++ +    ++  + +k   ++ + +++
NP_631558    115       GTDLVTLTIGGNDNstfinaitacgtagvlSGGKGSPCKDRHGTSFDDEI 164

                       efkdn..lrqlikrLrs.nngariivlitlvilnlg..........plG
                       e  +++ l++++  +r+++ +ar+ +l  ++i+++  +++     + +  G
NP_631558    165       EANTYpaLKEALLGVRArAPHARVAALGYPWITPATadpscflklplAAG 214

                       ClPlklalalassknvdasgclerlneavadfnealrelaiskledqlrk
                       P+                     l+ ++a  n a+r  a
NP_631558    215       DVPY----------------------LRAIQAHLNDAVRRAA--------- 234

                       dglpdvkgadvpyvDlysifqdldgiqnpsayvyGFettkaCCGyGgryN
                       ++ + +yvD+ ++
NP_631558    235       -------EETGATYVDFSGVSDG--------------------------- 250

                       ynrvCGnaglcnvtakaC.npssyll.sflfwDgf...HpsekGykavAe
                               ++aC+ p +++  +  lf + + + Hp++ G +++Ae
NP_631558    251       -------------HDACeAPGTRWIePLLFGHSLvpvHPNALGERRMAE 286

                       al<-*
                       +
NP_631558    287 HT    288
```

Alignment of pfam00657.6 consensus sequence with CAC42140

```
                    *->ivafGDSlTdgeayygdsdgggwgagladrLtallrlrarprgvdvf
                       +va+GDS ++g        +g + +++L      + + ++  +
CAC42140     42        YVALGDSYSAG---------SGVLPVDPANL----LCLRSTANYPHV 75

                       nrgisGrtsdGrlivD.a.l.vallFlaqslglpnLpPYLsgdflrGANF
                       + ++G++        D + + +
CAC42140     76        IADTTGAR-----LTDvTcGaAQ------------------------- 93

                       AsagAtIlptsgpfliQvqFkdfksqvlelrqalgllqellrllpvldak
                                              +++      ++ +  ++ +++
CAC42140     94        -----------------------TADFTRAQYPGVAPQLDALGT 114

                       spdlvtimiGtNDl................itsaffgpkstesdrnvsvp
                       + dlvt+ iG+ND ++  +  +  ++ +    ++  + +k   ++ + +++
CAC42140     115       GTDLVTLTIGGNDNstfinaitacgtagvlSGGKGSPCKDRHGTSFDDEI 164

                       efkdn..lrqlikrLrs.nngariivlitlvilnlg..........plG
                       e  +++ l++++  +r+++ +ar+ +l  ++i+++  +++    + +   G
CAC42140     165       EANTYpaLKEALLGVRArAPHARVAALGYPWITPATadpscflklplAAG 214

                       ClPlklalalassknvdasgclerlneavadfnealrelaiskledqlrk
                       P+                     l+ ++a  n a+r  a
CAC42140     215       DVPY----------------------LRAIQAHLNDAVRRAA--------- 234

                       dglpdvkgadvpyvDlysifqdldgiqnpsayvyGFettkaCCGyGgryN
                       ++ + +yvD+ ++
CAC42140     235       -------EETGATYVDFSGVSDG--------------------------- 250

                       ynrvCGnaglcnvtakaC.npssyll.sflfwDgf...HpsekGykavAe
                               ++aC+ p +++  +  lf + + + Hp++ G +++Ae
CAC42140     251       -------------HDACeAPGTRWIePLLFGHSLvpvHPNALGERRMAE 286
```

```
                        al<-*
                        +
         CAC42140   287 HT    288

Alignment of pfam00657.6 consensus sequence with P41734
                    *->ivafGDSlTdg....eayygdsdgggwgagladrLtallrlrarprg
                       ++fGDS+T+    +++ + +  d+    ga+l + +        +r+
         P41734     6  FLLFGDSITEFafntRPIEDGKDQYALGAALVMEY---------TRK 43

                       vdvfnrgisGrtsdGrlivDalvallFlaqslglpnLpPYLsgdflrGAN
                       +d+   rg++G+t
         P41734    44  MDILQRGFKGYT---------------------------------- 55

                       FAsagAtIlptsgpfliQvqFkdfksqvlelrqalgllqellrllpvlda
                                            +r+al++l+e+l+      +
         P41734    56  ----------------------------SRWALKILPEILKH-----E 70

                       kspdlvtimiGtNDlitsaffgpkstesdrnvsvpefkdnlrqlikrLrs
                       +  + ti++G+ND+       ++ +++ v++pef+dn+rq++++++s
         P41734    71  SNIVMATIFLGANDA---------CSAGPQSVPLPEFIDNIRQMVSLMKS 111

                       nngariivlitlvilnlgplGClPlklalalassknvdasgclerlneav
                       +++ii+++lv    ++              ++ k ++ +  + r+ne +
         P41734   112  YHIRPIIIGPGLVDREKW-----------EREKSEEIALGYFRTNENF 148

                       adfnealrelaiskledqlrkdglpdvkgadvpyvDlysifqdldgiqnp
                       a +   al +la          ++ +vp+v l+++fq+ +g++++
         P41734   149  AIYSDALAKLA---------------NEEKVPFVALNKAFQQEGGDAWQ 182

                       sayvyGFettkaCCGyGgryNynrvCGnaglcnvtakaCnpssyllsflf
                       +                                           1+
         P41734   183  Q---------------------------------------------LL 185

                       wDgfHpsekGykavAeal<-*
                       Dg+H+s kGyk++++l
         P41734   186  TDGLHFSGKGYKIFHDEL    203
```

Figure 8

```
A.sal   1  MKKWFVCLLGLIALTVQAADTRPAFSRIVMFGDSLSDTGKMYSKMRGYLPSSPPYYEGRF  60
              +           +
A.hyd   1  MKKWFVCLLGLVALTVQAADSRPAFSRIVMFGDSLSDTGKMYSKMRGYLPSSPPYYEGRF  60

A. sal  61  SNGPVWLEQLTKQFPGLTIANEAEGGATAVAYNKISWNPKYQVINNLDYEVTQFLQKDSF 120
                     ++          +
A. hyd  61  SNGPVWLEQLTNEFPGLTIANEAEGGPTAVAYNKISWNPKYQVINNLDYEVTQFLQKDSF 120

A. sal 121  KPDDLVILWVGANDYLAYGWNTEQDAKRVRDAISDAANRMVLNGAKQILLFNLPDLGQNP 180
                                                             +
A. hyd 121  KPDDLVILWVGANDYLAYGWNTEQDAKRVRDAISDAANRMVLNGAKEILLFNLPDLGQNP 180

A. sal 181  SARSQKVVEAVSHVSAYHNKLLLNLARQLAPTGMVKLFEIDKQFAEMLRDPQNFGLSDVE 240
                     +        +                                              ++
A.hyd  181  SARSQKVVEAASHVSAYHNQLLLNLARQLAPTGMVKLFEIDKQFAEMLRDPQNFGLSDQR 240

A. sal 241  NPCYDGGYVWKPFATRSVSTDRQLSAFSPQERLAIAGNPLLAQAVASPMARRSASPLNCE 300
              + ++ +       + +    +      +                          +    +
A. hyd 241  NACYGGSYVWKPFASRSASTDSQLSAFNPQERLAIAGNPLLAQAVASPMAARSASTLNCE 300

A. sal 301  GKMFWDQVHPTTVVHAALSERAATFIETQYEFLAH 335
                        +         +
A. hyd 301  GKMFWDQVHPTTVVHAALSEPAATFIESQYEFLAH 335
```

Figure 9

```
  1  ATGAAAAAAT GGTTTGTGTG TTTATTGGGA TTGGTCGCGC TGACAGTTCA GGCAGCCGAC
 61  AGCCGTCCCG CCTTCTCCCG GATCGTGATG TTTGGCGACA GCCTCTCCGA TACCGGCAAG
121  ATGTACAGCA AGATGCGCGG TTACCTCCCC TCCAGCCCCC CCTACTATGA GGGCCGCTTC
181  TCCAACGGGC CCGTCTGGCT GGAGCAGCTG ACCAACGAGT TCCCGGGCCT GACCATAGCC
241  AACGAGGCGG AAGGCGGACC GACCGCCGTG GCTTACAACA AGATCTCCTG GAATCCCAAG
301  TATCAGGTCA TCAACAACCT GGACTACGAG GTCACCCAGT TCCTGCAAAA AGACAGCTTC
361  AAGCCGGACG ATCTGGTGAT CCTCTGGGTC GGCGCCAACG ACTATCTGGC CTATGGCTGG
421  AACACAGAGC AGGATGCCAA GCGGGTGCGC GACGCCATCA GCGATGCGGC CAACCGCATG
481  GTGCTGAACG GCGCCAAGGA GATACTGCTG TTCAACCTGC CGGATCTGGG CCAGAACCCC
602  CTGCTGCTGA ACCTGGCACG CCAGCTGGCT CCCACCGGCA TGGTGAAGCT GTTCGAGATC
661  GACAAGCAGT TTGCCGAGAT GCTGCGTGAT CCGCAGAACT TCGGCCTGAG CGACCAGAGG
721  AACGCCTGCT ACGGTGGCAG CTATGTATGG AAGCCGTTTG CCTCCCGCAG CGCCAGCACC
781  GACAGCCAGC TCTCCGCCTT CAACCCGCAG GAGCGCCTCG CCATCGCCGG CAACCCGCTG
841  CTGGCCCAGG CCGTCGCCAG CCCCATGGCT GCCCGCAGCG CCAGCACCCT CAACTGTGAG
901  GGCAAGATGT TCTGGGATCA GGTCCACCCC ACCACTGTCG TGCACGCCGC CCTGAGCGAG
961  CCCGCCGCCA CCTTCATCGA GAGCCAGTAC GAGTTCCTCG CCCAC
```

Figure 10

```
  1  ATGAAAAAAT GGTTTGTTTG TTTATTGGGG TTGATCGCGC TGACAGTTCA GGCAGCCGAC
 61  ACTCGCCCCG CCTTCTCCCG GATCGTGATG TTCGGCGACA GCCTCTCCGA TACCGGCAAA
121  ATGTACAGCA AGATGCGCGG TTACCTCCCC TCCAGCCCGC CCTACTATGA GGGCCGTTTC
181  TCCAACGGAC CCGTCTGGCT GGAGCAGCTG ACCAAGCAGT TCCCGGGTCT GACCATCGCC
241  AACGAAGCGG AAGGCGGTGC CACTGCCGTG GCTTACAACA AGATCTCCTG GAATCCCAAG
301  TATCAGGTCT ACAACAACCT GGACTACGAG GTCACCCAGT TCTTGCAGAA AGACAGCTTC
361  AAGCCGGACG ATCTGGTGAT CCTCTGGGTC GGTGCCAATG ACTATCTGGC ATATGGCTGG
421  AATACGGAGC AGGATGCCAA GCGAGTTCGC GATGCCATCA GCGATGCGGC CAACCGCATG
481  GTACTGAACG GTGCCAAGCA GATACTGCTG TTCAACCTGC CGGATCTGGG CCAGAACCCG
541  TCAGCCCGCA GTCAGAAGGT GGTCGAGGCG GTCAGCCATG TCTCCGCCTA TCACAACAAG
601  CTGCTGCTGA ACCTGGCACG CCAGCTGGCC CCCACCGGCA TGGTAAAGCT GTTCGAGATC
661  GACAAGCAAT TTGCCGAGAT GCTGCGTGAT CCGCAGAACT TCGGCCTGAG CGACGTCGAG
721  AACCCCTGCT ACGACGGCGG CTATGTGTGG AAGCCGTTTG CCACCCGCAG CGTCAGCACC
781  GACCGCCAGC TCTCCGCCTT CAGTCCGCAG GAACGCCTCG CCATCGCCGG CAACCCGCTG
841  CTGGCACAGG CCGTTGCCAG TCCTATGGCC CGCCGCAGCG CCAGCCCCCT CAACTGTGAG
901  GGCAAGATGT TCTGGGATCA GGTACACCCG ACCACTGTCG TGCACGCAGC CCTGAGCGAG
961  CGCGCCGCCA CCTTCATCGA GACCCAGTAC GAGTTCCTCG CCCACGGATG A
```

Figure 11

```
  1  ATGCCGAAGC CTGCCCTTCG CCGTGTCATG ACCGCGACAG TCGCCGCCGT CGGCACGCTC
 61  GCCCTCGGCC TCACCGACGC CACCGCCCAC GCCGCGCCCG CCCAGGCCAC TCCGACCCTG
121  GACTACGTCG CCCTCGGCGA CAGCTACAGC GCCGGCTCCG GCGTCCTGCC CGTCGACCCC
181  GCCAACCTGC TCTGTCTGCG CTCGACGGCC AACTACCCCC ACGTCATCGC GGACACGACG
241  GGCGCCCGCC TCACGGACGT CACCTGCGGC GCCGCGCAGA CCGCCGACTT CACGCGGGCC
301  CAGTACCCGG GCGTCGCACC CCAGTTGGAC GCGCTCGGCA CCGGCACGGA CCTGGTCACG
361  CTCACCATCG GCGGCAACGA CAACAGCACC TTCATCAACG CCATCACGGC CTGCGGCACG
421  GCGGGTGTCC TCAGCGGCGG CAAGGGCAGC CCCTGCAAGG ACAGGCACGG CACCTCCTTC
481  GACGACGAGA TCGAGGCCAA CACGTACCCC GCGCTCAAGG AGGCGCTGCT CGGCGTCCGC
541  GCCAGGGCTC CCCACGCCAG GGTGGCGGCT CTCGGCTACC CGTGGATCAC CCCGGCCACC
601  GCCGACCCGT CCTGCTTCCT GAAGCTCCCC CTCGCCGCCG GTGACGTGCC CTACCTGCGG
661  GCCATCCAGG CACACCTCAA CGACGCGGTC CGGCGGGCCG CCGAGGAGAC CGGGAGCCACC
721  TACGTGGACT TCTCCGGGGT GTCCGACGGC CACGACGCCT GCGAGGCCCC CGGCACCCGC
781  TGGATCGAAC CGCTGCTCTT CGGGCACAGC CTCGTTCCCG TCCACCCCAA CGCCCTGGGC
841  GAGCGGCGCA TGGCCGAGCA CACGATGGAC GTCCTCGGCC TGGACTGA
```

Figure 12

```
  1  TCAGTCCAGG CCGAGGACGT CCATCGTGTG CTCGGCCATG CGCCGCTCGC CCAGGGCGTT
 61  GGGGTGGACG GGAACGAGGC TGTGCCCGAA GAGCAGCGGT TCGATCCAGC GGGTGCCGGG
121  GGCCTCGCAG GCGTCGTGGC CGTCGGACAC CCCGGAGAAG TCCACGTAGG TGGCTCCGGT
181  CTCCTCGGCG GCCCGCCGGA CCGCGTCGTT GAGGTGTGCC TGGATGGCCC GCAGGTAGGG
241  CACGTCACCG GCGGCGAGGG GGAGCTTCAG GAAGCAGGAC GGGTCGGCGG TGGCCGGGGT
301  GATCCACGGG TAGCCGAGAG CCGCCACCCT GGCGTGGGGA GCCCTGGCGC GGACGCCGAG
361  CAGCGCCTCC TTGAGCGCGG GGTACGTGTT GGCCTCGATC TCGTCGTCGA AGGAGGTGCC
421  GTGCCTGTCC TTGCAGGGGC TGCCCTTGCC GCCGCTGAGG ACACCCGCCG TGCCGCAGGC
481  CGTGATGGCG TTGATGAAGG TGCTGTTGTC GTTGCCGCCG ATGGTGAGCG TGACCAGGTC
541  CGTGCCGGTG CCGAGCGCGT CCAACTGGGG TGCGACGCCC GGGTACTGGG CCCGCGTGAA
601  GTCGGCGGTC TGCGCGGCGC CGCAGGTGAC GTCCGTGAGG CGGGCGCCCG TCGTGTCCGC
661  GATGACGTGG GGGTAGTTGG CCGTCGAGCG CAGACAGAGC AGGTTGGCGG GGTCGACGGG
721  CAGGACGCCG GAGCCGGCGC TGTAGCTGTC GCCGAGGGCG ACGTAGTCCA GGGTCGGAGT
781  GGCCTGGGCG GGCGCGGCGT GGGCGGTGGC GTCGGTGAGG CCGAGGGCGA GCGTGCCGAC
841  GGCGGCGACT GTCGCGGTCA TGACACGGCG AAGGGCAGGC TTCGGCAT
```

Figure 13

```
  1  ATGGATTACG AGAAGTTTCT GTTATTTGGG GATTCCATTA CTGAATTTGC TTTTAATACT
 61  AGGCCCATTG AAGATGGCAA AGATCAGTAT GCTCTTGGAG CCGCATTAGT CAACGAATAT
121  ACGAGAAAAA TGGATATTCT TCAAAGAGGG TTCAAAGGGT ACACTTCTAG ATGGGCGTTG
181  AAAATACTTC CTGAGATTTT AAAGCATGAA TCCAATATTG TCATGGCCAC AATATTTTTG
241  GGTGCCAACG ATGCATGCTC AGCAGGTCCC CAAAGTGTCC CCCTCCCCGA ATTTATCGAT
301  AATATTCGTC AAATGGTATC TTTGATGAAG TCTTACCATA TCCGTCCTAT TATAATAGGA
361  CCGGGGCTAG TAGATAGAGA GAAGTGGGAA AAAGAAAAAT CTGAAGAAAT AGCTCTCGGA
421  TACTTCCGTA CCAACGAGAA CTTTGCCATT TATTCCGATG CCTTAGCAAA ACTAGCCAAT
481  GAGGAAAAAG TTCCCTTCGT GGCTTTGAAT AAGGCGTTTC AACAGGAAGG TGGTGATGCT
541  TGGCAACAAC TGCTAACAGA TGGACTGCAC TTTTCCGGAA AAGGGTACAA AATTTTTCAT
601  GACGAATTAT TGAAGGTCAT TGAGACATTC TACCCCCAAT ATCATCCCAA AAACATGCAG
661  TACAAACTGA AAGATTGGAG AGATGTGCTA GATGATGGAT CTAACATAAT GTCTTGA
```

Figure 14

(SEQ ID No. 12)

```
         10         20         30         40         50         60
          |          |          |          |          |          |
MNLRQWMGAA TAALALGLAA CGGGGTDQSG NPNVAKVQRM VVFGDSLSDI GTYTPVAQAV


         70         80         90        100        110        120
          |          |          |          |          |          |
GGGKFTTNPG PIWAETVAAQ LGVTLTPAVM GYATSVQNCP KAGCFDYAQG GSRVTDPNGI


        130        140        150        160        170        180
          |          |          |          |          |          |
GHNGGAGALT YPVQQQLANF YAASNNTFNG NNDVVFVLAG SNDIFFWTTA AATSGSGVTP


        190        200        210        220        230        240
          |          |          |          |          |          |
AIATAQVQQA ATDLVGYVKD MIAKGATQVY VFNLPDSSLT PDGVASGTTG QALLRALVGT


        250        260        270        280        290        300
          |          |          |          |          |          |
FNTTLQSGLA GTSARIIDFN AQLTAAIQNG ASFGFANTSA RACDATKINA LVPSAGGSSL


        310        320        330        340
          |          |          |          |
FCSANTLVAS GADQSYLFAD GVHPTTAGHR LIASNVLARL LADNVAH
```

Figure 15

(SEQ ID No. 13)

```
atgaacctgc gtcaatggat gggcgccgcc acggctgccc ttgccttggg cttggccgcg    60
tgcggggggcg gtgggaccga ccagagcggc aatcccaatg tcgccaaggt gcagcgcatg   120
gtggtgttcg gcgacagcct gagcgatatc ggcacctaca ccccgtcgc gcaggcggtg    180
ggcggcggca agttcaccac caacccgggc ccgatctggg ccgagaccgt ggccgcgcaa   240
ctgggcgtga cgctcacgcc ggcggtgatg ggctacgcca cctccgtgca gaattgcccc   300
aaggccggct gcttcgacta tgcgcagggc ggctcgcgcg tgaccgatcc gaacggcatc   360
ggccacaacg gcggcgcggg ggcgctgacc tacccggttc agcagcagct cgccaacttc   420
tacgcggcca gcaacaacac attcaacggc aataacgatg tcgtcttcgt gctggccggc    480
agcaacgaca ttttcttctg gaccactgcg gcggccacca gcggctccgg cgtgacgccc   540
gccattgcca cggcccaggt gcagcaggcc gcgacggacc tggtcggcta tgtcaaggac   600
atgatcgcca agggtgcgac gcaggtctac gtgttcaacc tgcccgacag cagcctgacg   660
ccggacggcg tggcaagcgg cacgaccggc caggcgctgc tgcacgcgct ggtgggcacg   720
ttcaacacga cgctgcaaag cgggctggcc ggcacctcgg cgcgcatcat cgacttcaac   780
gcacaactga ccgcggcgat ccagaatggc gcctcgttcg gcttcgccaa caccagcgcc   840
cgggcctgcg acgccaccaa gatcaatgcc ctggtgccga gcgccggcgg cagctcgctg   900
ttctgctcgg ccaacacgct ggtggcttcc ggtgcggacc agagctacct gttcgccgac   960
ggcgtgcacc cgaccacggc cggccatcgc ctgatcgcca gcaacgtgct ggcgcgcctg  1020
ctggcggata acgtcgcgca ctga                                         1044
```

Figure 16 (SEQ ID No. 20)

```
  1 migsyvavgd sftegvgdpg pdgafvgwad rlavlladrr pegdftytnl avrgrlldqi
 61 vaeqvprvvg lapdlvsfaa ggndiirpgt dpdevaerfe lavaaltaaa gtvlvttgfd
121 trgvpvlkhl rgkiatyngh vraiadrygc pvldlwslrs vqdrrawdad rlhlspeght
181 rvalragqal glrvpadpdq pwpplpprgt ldvrrddvhw areylvpwig rrlrgessgd
241 hvtakgtlsp daiktriaav a
```

Figure 17 (SEQ ID No. 21)

```
  1 gtgatcgggt cgtacgtggc ggtgggggac agcttcaccg agggcgtcgg cgaccccggc
 61 cccgacgggg cgttcgtcgg ctgggccgac cggctcgccg tactgctcgc ggaccggcgc
121 cccgagggcg acttcacgta cacgaacctc gccgtgcgcg gcaggctcct cgaccagatc
181 gtggcggaac aggtcccgcg ggtcgtcgga ctcgcgcccg acctcgtctc gttcgcggcg
241 ggcggcaacg acatcatccg gcccggcacc gatcccgacg aggtcgccga gcggttcgag
301 ctggcggtgg ccgcgctgac cgccgcgggcc ggaaccgtcc tggtgaccac cgggttcgac
361 acccggggggg tgcccgtcct caagcacctg cgcggcaaga tcgccacgta caacgggcac
421 gtccgcgcca tcgccgaccg ctacggctgc ccggtgctcg acctgtggtc gctgcggagc
481 gtccaggacc gcagggcgtg ggacgccgac cggctgcacc tgtcgccgga ggggcacacc
541 cgggtggcgc tgcgcgcggg gcaggccctg ggcctgcgcg tcccggccga ccctgaccag
601 ccctggccgc ccctgccgcc gcgcggcacg ctcgacgtcc ggcgcgacga cgtgcactgg
661 gcgcgcgagt acctggtgcc gtggatcggg cgccggctgc ggggcgagtc gtcgggcgac
721 cacgtgacgg ccaaggggac gctgtcgccg gacgccatca agacgcggat cgccgcggtg
781 gcctga
```

Figure 18
(SEQ ID No. 22)

```
  1 mqtnpaytsl vavgdsfteg msdllpdgsy rgwadllatr maarspgfry anlavrgkli
 61 gqivdeqvdv aaamgadvit lvgglndtlr pkcdmarvrd lltqaverla phceqlvlmr
121 spgrqgpvle rfrprmealf aviddlagrh gavvvdlyga qsladprmwd vdrlhltaeg
181 hrrvaeavwq slghepedpe whapipatpp pgwvtrrtad vrfarqhllp wigrrltgrs
241 sgdglpakrp dllpyedpar
```

Figure 19 (SEQ ID No. 23)

```
  1 atgcagacga accccgcgta caccagtctc gtcgccgtcg gcgactcctt caccgagggc
 61 atgtcggacc tgctgcccga cggctcctac cgtggctggg ccgacctcct cgccacccgg
121 atggcggccc gctcccccgg cttccggtac gccaacctgg cggtgcgcgg gaagctgatc
181 ggacagatcg tcgacgagca ggtggacgtg gccgccgcca tgggagccga cgtgatcacg
241 ctggtcggcg ggctcaacga cacgctgcgg cccaagtgcg acatggcccg ggtgcgggac
301 ctgctgaccc aggccgtgga acggctcgcc ccgcactgcg agcagctggt gctgatgcgc
361 agtcccggtc gccagggtcc ggtgctggag cgcttccggc cccgcatgga ggccctgttc
421 gccgtgatcg acgacctggc cgggcggcac ggcgccgtgg tcgtcgacct gtacggggcc
481 cagtcgctgg ccgaccctcg gatgtgggac gtggaccggc tgcacctgac cgccgagggc
541 caccgccggg tgcggaggc ggtgtggcag tcgctcggcc acgagcccga ggaccccgag
601 tggcacgcgc cgatcccggc gacgccgccg ccggggtggg tgacgcgcag gaccgccggac
661 gtccggttcg cccggcagca cctgctgccc tggataggcc gcaggctgac cgggcgctcg
721 tccggggacg gcctgccggc caagcgcccg gacctgctgc cctacgagga cccgcacgg
781 tga
```

Figure 20 (SEQ ID No. 24)

```
  1 mtrgrdggag apptkhrall aaivtlivai saaiyagasa ddgsrdhalq aggrlprgda
 61 apastgawvg awatapaaae pgtettglag rsvrnvvhts vggtgaritl snlygqsplt
121 vthasialaa gpdtaaaiad tmrrltfggs arviipaggq vmsdtarlai pyganvlvtt
181 yspipsgpvt ybpqarqtsy ladgdrtadv tavayttptp ywryltaldv lsheadgtvv
241 afgdsitdga rsqsdanhrw tdvlaarlhe aagdgrdtpr ysvvnegisg nrlltsrpgr
301 padnpsglsr fqrdvlertn vkavvvvlgv ndvlnspela drdailtglr tlvdraharg
361 lrvvgatitp fggyggytea retmrqevne eirsgrvfdt vvdfdkalrd pydprrmrsd
421 ydsgdhlhpg dkgyarmgav idlaalkgaa pvka
```

Figure 21 (SEQ ID No. 25)

```
   1 atgacccggg gtcgtgacgg gggtgcgggg gcgcccccca ccaagcaccg tgccctgctc
  61 gcggcgatcg tcaccctgat agtggcgatc tccgcggcca tatacgccgg agcgtccgcg
 121 gacgacggca gcagggacca cgcgctgcag gccggaggcc gtctcccacg aggagacgcc
 181 gcccccgcgt ccaccggtgc ctgggtgggc gcctgggcca ccgcaccggc cgcggccgag
 241 ccgggcaccg agacgaccgg cctggcgggc cgctccgtgc gcaacgtcgt gcacacctcg
 301 gtcggcggca ccggcgcgcg gatcaccctc tcgaacctgt acgggcagtc gccgctgacc
 361 gtcacacacg cctcgatcgc cctggccgcc gggcccgaca ccgccgcgcg gatcgccgac
 421 accatgcgcc ggctcacctt cggcggcagc gcccgggtga tcatcccggc gggcggccag
 481 gtgatgagcg acaccgcccg cctcgccatc ccctacgggg cgaacgtcct ggtcaccacg
 541 tactcccca tcccgtccgg gccggtgacc taccatccgc aggcccggca gaccagctac
 601 ctggccgacg gcgaccgcac ggcggacgtc accgccgtcg cgtacaccac ccccacgccc
 661 tactggcgct acctgaccgc cctcgacgtg ctgagccacg aggccgacgg cacggtcgtg
 721 gcgttcggcg actccatcac cgacggcgcc cgctcgcaga gcgacgccaa ccaccgctgg
 781 accgacgtcc tcgccgcacg cctgcacgag gcggcgggcg acggccggga cacgcccgc
 841 tacagcgtcg tcaacgaggg catcagcggc aaccggctcc tgaccagcag gccggggcgg
 901 ccggccgaca acccgagcgg actgagccgg ttccagcggg acgtgctgga acgcaccaac
 961 gtcaaggccg tcgtcgtcgt cctcggcgtc aacgacgtcc tgaacagccc ggaactcgcc
1021 gaccgcgacg ccatcctgac cggcctgcgc accctcgtcg accgggcgca cgcccgggga
1081 ctgcgggtcg tcggcgccac gatcacgccg ttcggcgggct acggcggcta caccgaggcc
1141 cgcgagacga tgcggcagga ggtcaacgag gagatccgct ccggccgggt cttcgacacg
1201 gtcgtcgact tcgacaaggc cctgcgcgac ccgtacgacc cgcgccggat gcgctccgac
1261 tacgacagcg gcgaccacct gcaccccggc gacaaggggt acgcgcgcat gggcgcggtc
1321 atcgacctgg ccgcgctgaa gggcgcggcg ccggtcaagg cgtag
```

Figure 22 (SEQ ID No. 26)

```
  1 mtsmsrarva rrlaagaayg gggiglagaa avglvvaevq larrrvgvgt ptrvpnaqgl
 61 yggtlptagd pplrlmmlgd staagqgvhr agqtpgalla sglaavaerp vrlgsvaqpg
121 acsddldrqv alvlaepdrv pdicvimvga ndvthrmpat rsvrhlssav rrlrtagaev
181 vvgtcpdlgt iervrqplrw larrasrqla aaqtigaveq ggrtvslgdl lgpefaqnpr
241 elfgpdnyhp saegyataam avlpsvcaal glwpadeehp dalrregflp varaaaeaas
301 eagtevaaam ptgprgpwal lkrrrrrrvs eaepsspsgv
```

Figure 23 (SEQ ID No. 27)

```
   1 atgacgagca tgtcgagggc gagggtggcg cggcggatcg cggccggcgc ggcgtacggc
  61 ggcggcggca tcggcctggc gggagcggcg gcggtcggtc tggtggtggc cgaggtgcag
 121 ctggccagac gcagggtggg ggtgggcacg ccgacccggt tgccgaacgc gcagggactg
 181 tacggcggca ccctgcccac ggccggcgac ccgccgctgc ggctgatgat gctgggcgac
 241 tccacggccg ccgggcaggg cgtgcaccgg gccgggcaga cgccgggcgc gctgctggcg
 301 tccgggctcg cggcggtggc ggagcggccg gtgcggctgg ggtcggtcgc ccagccgggg
 361 gcgtgctcgg acgacctgga ccggcaggtg gcgctggtgc tcgccgagcc ggaccgggtg
 421 cccgacatct gcgtgatcat ggtcggcgcc aacgacgtca cccaccggat gccggcgacc
 481 cgctcggtgc ggcacctgtc ctcggcggta cggcggctgc gcacggccgg tgcggaggtg
 541 gtggtcggca cctgtccgga cctgggcacg atcgagcggg tgcggcagcc gctgcgctgg
 601 ctggcccggc gggcctcacg gcagctcgcg gcggcacaga ccatcggcgc cgtcgagcag
 661 ggcgggcgca cggtgtcgct gggcgacctg ctgggtccgg agttcgcgca gaacccgcgg
 721 gagctcttcg gccccgacaa ctaccacccc tccgccgagg ggtacgccac ggccgcgatg
 781 gcggtactgc cctcggtgtg cgccgcgctc ggcctgtggc cggccgacga ggagcacccg
 841 gacgcgctgc gccgcgaggg cttcctgccg gtggcgcgcg cggcggcgga ggcggcgtcc
 901 gaggcgggta cggaggtcgc cgccgccatg cctacgggc ctcggggcc ctgggcgctg
 961 ctgaagcgcc ggagacggcg tcgggtgtcg gaggcggaac cgtccagccc gtccggcgtt
1021 tga
```

Figure 24 (SEQ ID No. 28)

```
  1 mgrgtdqrtr ygrrrarval aaltaavlgv gvagcdsvgg dspapsgsps krtrtapawd
 61 tspasvaavg dsitrgfdac avlsdcpevs watgssakvd slavrllgka daaehswnya
121 vtgarmadlt aqvtraaqre pelvavmaga ndacrsttsa mtpvadfraq feeamatlrk
181 klpkaqvyvs sipdlkrlws qgrtnplgkq vwklglcpsm lgdadsldsa atlrrntvrd
241 rvadynevlr evcakdrrcr sddgavhefr fgtdqlshwd wfhpsvdgqa rlaeiayrav
301 taknp
```

Figure 25 (SEQ ID No. 29)

```
  1 atgggtcgag ggacggacca gcggacgcgg tacggccgtc gccgggcgcg tgtcgcgctc
 61 gccgccctga ccgccgccgt cctgggcgtg ggcgtggcgg gctgcgactc cgtgggcggc
121 gactcacccg ctccttccgg cagcccgtcg aagcggacga ggacggcgcc cgcctgggac
181 accagcccgg cgtccgtcgc cgccgtgggc gactccatca cgcgcggctt cgacgcctgt
241 gcggtgctgt cggactgccc ggaggtgtcg tggcgaccg gcagcagcgc gaaggtcgac
301 tcgctggccg tacggctgct ggggaaggcg gacgcggccg agcacagctg gaactacgcg
361 gtcaccgggg cccggatggc ggacctgacc gctcaggtga cgcgggcggc gcagcgcgag
421 ccggagctgg tggcggtgat ggccgggggcg aacgacgcgt gccggtccac gacctcggcg
481 atgacgccgg tggcggactt ccgggcgcag ttcgaggagg cgatggccac cctgcgcaag
541 aagctcccca aggcgcaggt gtacgtgtcg agcatcccgg acctcaagcg gctctggtcc
601 cagggccgca ccaacccgct gggcaagcag gtgtggaagc tcggcctgtg cccgtcgatg
661 ctgggcgacg cggactccct ggactcggcg gcgaccctgc ggcgcaacac ggtgcgcgac
721 cgggtggcgg actacaacga ggtgctgcgg gaggtctgcg cgaaggaccg gcggtgccgc
781 agcgacgacg gcgcggtgca cgagttccgg ttcggcacgg accagttgag ccactgggac
841 tggttccacc cgagtgtgga cggccaggcc cggctggcgg agatcgccta ccgcgcggtc
901 accgcgaaga atccctga
```

Figure 26 (SEQ ID No. 30)

```
  1 mrlsrraata sallltpala lfgasaavsa priqatdyva lgdsyssgvg agsydsssgs
 61 ckrstksypa lwaashtgtr fnftacsgar tgdvlakqlt pvnsgtdlvs itiggndagf
121 adtmttcnlq gesaclaria karayiqqtl paqldqvyda idsrapaaqv vvlgyprfyk
181 lggscavgls eksraainaa addinavtak raadhgfafg dvnttfaghe lcsgapwlhs
241 vtlpvensyh ptangqskgy lpvlnsat
```

Figure 27 (SEQ ID No. 31)

```
   1 ttcatcacaa cgatgtcaca acaccggcca tccgggtcat ccctgatcgt gggaatgggt
  61 gacaagcctt cccgtgacga aagggtcctg ctacatcaga aatgacagaa atcctgctca
 121 gggaggttcc atgagactgt cccgacgcgc ggccacggcg tccgcgctcc tcctcaccoc
 181 ggcgctcgcg ctcttcggcg cgagcgccgc cgtgtccgcg ccgcgaatcc aggccaccga
 241 ctacgtggcc ctcggcgact cctactcctc gggggtcggc gcgggcagct acgacagcag
 301 cagtggctcc tgtaagcgca gcaccaagtc ctacccggcc ctgtgggccg cctcgcacac
 361 cggtacgcgg ttcaacttca ccgcctgttc gggcgcccgc acaggagacg tgctggccaa
 421 gcagctgacc ccggtcaact ccggcaccga cctggtcagc attaccatcg gcggcaacga
 481 cgcgggcttc gccgacacca tgaccacctg caacctccag ggcgagagcg cgtgcctggc
 541 gcggatcgcc aaggcgcgcg cctacatcca gcagacgctg cccgcccagc tggaccaggt
 601 ctacgacgcc atcgacagcc gggcccccgc agcccaggtc gtcgtcctgg gctacccgcg
 661 cttctacaag ctgggcggca gctgcgccgt cggtctctcg gagaagtccc gcgcggccat
 721 caacgccgcc gccgacgaca tcaacgccgt caccgccaag cgcgccgccg accacggctt
 781 cgccttcggg gacgtcaaca cgaccttcgc cgggcacgag ctgtgctccg gcgcccctg
 841 gctgcacagc gtcacccttc ccgtggagaa ctcctaccac cccacggcca acggacagtc
 901 caagggctac ctgcccgtcc tgaactccgc cacctgatct cgcggctact ccgcccctga
 961 cgaagtcccg cccccgggcg gggcttcgcc gtaggtgcgc gtaccgccgt cgcccgtcgc
1021 gccggtggcc ccgccgtacg tgccgccgcc cccggacgcg gtcggttc
```

Figure 28 (SEQ ID No. 32)

```
  1   MKKWFVCLLG LVALTVQAAD SRPAFSRIVM FGDSLSDTGK MYSKMRGYLP
 51   SSPPYYEGRF SWGPVWLEQL TKQFPGLTIA NEAEGGATAV AYNKISWNPK
101   YQVINNLDYE VTQFLQRDSF KPDDLVILWV GANDYLAYGW NTEQDARRVR
151   DAISDAANRM VLNGAKQILL FNLPDLGQNP SARSQKVVEA VSHVSAYHNQ
201   LLLNLARQLA PTGMVKLFEI DKQFAEMLRD PQNFGLSDVE NPCYDGGYVW
251   KPFATRSVST DRQLSAFSPQ ERLAIAGNPL LAQAVASPMA RRSASPLNCE
301   GKMFWDQVHP TTVVHAALSE RAATFIANQY EFLAH*
```

Figure 29 (SEQ ID No. 33)

```
   1   ATGAAAAAAT GGTTTGTGTG TTTATTGGGA TTGGTCGCGC TGACAGTTCA
       TACTTTTTTA CCAAACACAC AAATAACCCT AACCAGCGCG ACTGTCAAGT

  51   GGCAGCCGAC AGTCGCCCCG CCTTTTCCCG GATCGTGATG TTCGGCGACA
       CCGTCGGCTG TCAGCGGGGC GGAAAAGGGC CTAGCACTAC AAGCCGCTGT

 101   GCCTCTCCGA TACCGGCAAA ATGTACAGCA AGATGCGCGG TTACCTCCCC
       CGGAGAGGCT ATGGCCGTTT TACATGTCGT TCTACGCGCC AATGGAGGGG

 151   TCCAGCCCGC CCTACTATGA GGGCCGTTTC TCCAACGGAC CCGTCTGGCT
       AGGTCGGGCG GGATGATACT CCCGGCAAAG AGGTTGCCTG GGCAGACCGA

 201   GGAGCAGCTG ACCAAACAGT TCCCGGGTCT GACCATCGCC AACGAAGCGG
       CCTCGTCGAC TGGTTTGTCA AGGGCCCAGA CTGGTAGCGG TTGCTTCGCC

 251   AAGGCGGTGC CACTGCCGTG GCTTACAACA AGATCTCCTG GAATCCCAAG
       TTCCGCCACG GTGACGGCAC CGAATGTTGT TCTAGAGGAC CTTAGGGTTC

 301   TATCAGGTCA TCAACAACCT GGACTACGAG GTCACCCAGT TCTTGCAGAA
       ATAGTCCAGT AGTTGTTGGA CCTGATGCTC CAGTGGGTCA AGAACGTCTT

 351   AGACAGCTTC AAGCCGGACG ATCTCGTGAT CCTCTGGGTC GGTGCCAATG
       TCTGTCGAAG TTCGGCCTGC TAGACCACTA GGAGACCCAG CCACGGTTAC

 401   ACTATCTGGC CTATGGCTGG AACACGGAGC AGGATGCCAA GCGGGTTCGC
       TGATAGACCG GATACCGACC TTGTGCCTCG TCCTACGGTT CGCCCAAGCG

 451   GATGCCATCA GCGATGCGGC CAACCGCATG GTACTGAACG GTGCCAAGCA
       CTACGGTAGT CGCTACGCCG GTTGGCGTAC CATGACTTGC CACGGTTCGT

 501   GATACTGCTG TTCAACCTGC CGGATCTGGG CCAGAACCCG TCAGCTCGCA
       CTATGACGAC AAGTTGGACG GCCTAGACCC GGTCTTGGGC AGTCGAGCGT

 551   GTCAGAAGGT GGTCGAGGCG GTCAGCCATG TCTCCGCCTA TCACAACCAG
       CAGTCTTCCA CCAGCTCCGC CAGTCGGTAC AGAGGCGGAT AGTGTTGGTC

 601   CTGCTGCTGA ACCTGGCACG CCAGCTGGCC CCCACCGGCA TGGTAAAGCT
       GACGACGACT TGGACCGTGC GGTCGACCGG GGGTGGCCGT ACCATTTCGA

 651   GTTCGAGATC GACAAGCAAT TTGCCGAGAT GCTGCGTGAT CCGCAGAACT
       CAAGCTCTAG CTGTTCGTTA AACGGCTCTA CGACGCACTA GGCGTCTTGA

 701   TCGGCCTGAG CGACGTCGAG AACCCCTGCT ACGACGGCGG CTATGTGTGG
       AGCCGGACTC GCTGCAGCTC TTGGGACGA TGCTGCCGCC GATACACACC

 751   AAGCCGTTTG CCACCCGCAG CGTCAGCACC GACCGCCAGC TCTCCGCCTT
       TTCGGCAAAC GGTGGGCGTC GCAGTCGTGG CTGGCGGTCG AGAGGCGGAA

 801   CAGTCCGCAG GAACGCCTCG CCATCGCCGG CAACCCGCTG CTGGCACAGG
       GTCAGGCGTC CTTGCGGAGC GGTAGCGGCC GTTGGGCGAC GACCGTGTCC

 851   CCGTTGCCAG TCCTATGGCC CGCCGCAGCG CCAGCCCCCT CAACTGTGAG
       GGCAACGGTC AGGATACCGG GCGGCGTCGC GGTCGGGGGA GTTGACACTC

 901   GGCAAGATGT TCTGGGATCA GGTACACCCG ACCACTGTCG TGCACGCAGC
       CCGTTCTACA AGACCCTAGT CCATGTGGGC TGGTGACAGC ACGTGCGTCG

 951   CCTGAGCGAG CGCGCCGCCA CCTTCATCGC GAACCAGTAC GAGTTCCTCG
       GGACTCGCTC GCGCGGCGGT GGAAGTAGCG CTTGGTCATG CTCAAGGAGC

1001   CCCAC TGA
       GGGTG ACT
```

Figure 30 (SEQ ID No. 34)

```
  1  MKKWFVCLLG LIALTVQAAD TRPAPSRIVM FGDSLSDTGK MYSKMRGYLP
 51  SSPPYYEGRF SNGPVWLEQL TKQFPGLTIA NEAEGGATAV AYNKISWNPK
101  YQVINNLDYE VTQFLQKDSF KPDDLVILWV GANDYLAYGW NTEQDAKRVR
151  DAISDAANRM VLNGAKQILL FNLPDLGQNP SARSQKVVEA VSHVSAYHNK
201  LLLNLARQLA PTGMVKLFEI DKQFAEMLRD PQNFGLSDVE NPCYDGGYVW
251  KPFATRSVST DRQLSAFSPQ ERLAIAGNPL LAQAVASPMA RRSASPLNCE
301  GKMFWDQVHP TTVVHAALSE RAATFIETQY EFLAHG*
```

Figure 31 (SEQ ID No. 35)

```
   1  ATGAAAAAAT GGTTTGTTTG TTTATTGGGG TTGATCGCGC TGACAGTTCA
      TACTTTTTTA CCAAACAAAC AAATAACCCC AACTAGCGCG ACTGTCAAGT

  51  GGCAGCCGAC ACTCGCCCCG CCTTCTCCCG GATCGTGATG TTCGGCGACA
      CCGTCGGCTG TGAGCGGGGC GGAAGAGGGC CTAGCACTAC AAGCCGCTGT

 101  GCCTCTCCGA TACCGGCAAA ATGTACAGCA AGATGCGCGG TTACCTCCCC
      CGGAGAGGCT ATGGCCGTTT TACATGTCGT TCTACGCGCC AATGGAGGGG

 151  TCCAGCCCGC CCTACTATGA GGGCCGTTTC TCCAACGGAC CCGTCTGGCT
      AGGTCGGGCG GGATGATACT CCCGGCAAAG AGGTTGCCTG GCAGACCGA

 201  GGAGCAGCTG ACCAAGCAGT TCCCGGGTCT GACCATCGCC AACGAAGCGG
      CCTCGTCGAC TGGTTCGTCA AGGGCCCAGA CTGGTAGCGG TTGCTTCGCC

 251  AAGGCGGTGC CACTGCCGTG GCTTACAACA AGATCTCCTG GAATCCCAAG
      TTCCGCCACG GTGACGGCAC CGAATGTTGT TCTAGAGGAC CTTAGGGTTC

 301  TATCAGGTCA TCAACAACCT GGACTACGAG GTCACCCAGT TCTTGCAGAA
      ATAGTCCAGT AGTTGTTGGA CCTGATGCTC CAGTGGGTCA AGAACGTCTT

 351  AGACAGCTTC AAGCCGGACG ATCTGGTGAT CCTCTGGGTC GGTGCCAATG
      TCTGTCGAAG TTCGGCCTGC TAGACCACTA GGAGACCCAG CCACGGTTAC

 401  ACTATCTGGC ATATGGCTGG AATACGGAGC AGGATGCCAA GCGAGTTCGC
      TGATAGACCG TATACCGACC TTATGCCTCG TCCTACGGTT CGCTCAAGCG

 451  GATGCCATCA GCGATGCGGC CAACCGCATG GTACTGAACG GTGCCAAGCA
      CTACGGTAGT CGCTACGCCG GTTGGCGTAC CATGACTTGC CACGGTTCGT

 501  GATACTGCTG TTCAACCTGC CGGATCTGGG CCAGAACCCG TCAGCCCGCA
      CTATGACGAC AAGTTGGACG GCCTAGACCC GGTCTTGGGC AGTCGGGCGT

 551  GTCAGAAGGT GGTCGAGGCG GTCAGCCATG TCTCCGCCTA TCACAACAAG
      CAGTCTTCCA CCAGCTCCGC CAGTCGGTAC AGAGGCGGAT AGTGTTGTTC

 601  CTGCTGCTGA ACCTGGCACG CCAGCTGGCC CCCACCGGCA TGGTAAAGCT
      GACGACGACT TGGACCGTGC GGTCGACCGG GGGTGGCCGT ACCATTTCGA

 651  GTTCGAGATC GACAAGCAAT TTGCCGAGAT GCTGCGTGAT CCGCAGAACT
      CAAGCTCTAG CTGTTCGTTA AACGGCTCTA CGACGCACTA GGCGTCTTGA

 701  TCGGCCTGAG CGACGTCGAG AACCCCTGCT ACGACGGCGG CTATGTGTGG
      AGCCGGACTC GCTGCAGCTC TTGGGGACGA TGCTGCCGCC GATACACACC

 751  AAGCCGTTTG CCACCCGCAG CGTCAGCACC GACCGCCAGC TCTCCGCCTT
      TTCGGCAAAC GGTGGCCGTC GCAGTCGTGG CTGGCGGTCG AGAGGCGGAA

 801  CAGTCCGCAG GAACGCCTCG CCATCGCCGG CAACCCGCTG CTGGCACAGG
      GTCAGGCGTC CTTGCGGAGC GGTAGCGGCC GTTGGGCGAC GACCGTGTCC

 851  CCGTTGCCAG TCCTATGGCC CGCCGCAGCG CCAGCCCCCT CAACTGTGAG
      GGCAACGGTC AGGATACCGG GCGGCGTCGC GGTCGGGGGA GTTGACACTC

 901  GGCAAGATGT TCTGGGATCA GGTACACCCG ACCACTGTCG TGCACGCAGC
      CCGTTCTACA AGACCCTAGT CCATGTGGGC TGGTGACAGC ACGTGCGTCG

 951  CCTGAGCGAG CGCGCCGCCA CCTTCATCGA GACCCAGTAC GAGTTCCTCG
      GGACTCGCTC GCGCGGCGGT GGAAGTAGCT CTGGGTCATG CTCAAGGAGC

1001  CCCACGGATG A
      GGGTGCCTAC T
```

Figure 32

```
            1         10        20        30        40        50
            |-----------+---------+---------+---------+---------|
   satA     ADTRPAFSRIVNFGDSLSDTGKNYSKNRGYLPSSPPYYEGRFSN---G
   R.sol    QSGNPNVAKVQRNVYFGDSLSDIGT----------YTPVAQAYGGGKFTTNPG
   Consensus ...adnraafqRiVnFGDSLSDiGk........YlPsaqaygeGrFsn..G

            51        60        70        80        90        100
            |-----------+---------+---------+---------+---------|
   satA     PVNLEQLTKQFPGLTIANEAEGGATAVAYNKISNNPKYQVINNLDYEVTQ
   R.sol    PINRETVAAQL-GVTLTPAVHGYATSVQNCPKAGCFDYAQGGSRVTDPNG
   Consensus P!NaEqlaaQl.GlTianaaeGgATaVannkiagnfdYaqgnnrdt.#pnq

            101       110       120       130       140       150
            |-----------+---------+---------+---------+---------|
   satA     FLQKDSFKPDDLVILNVGANDYLAYG---KNTEQDAKRVRDAISDAANRNV
   R.sol    IGHNGGAGALTYPVQQQLANFYAASNNTFNGNNDVVFVLAGSNDIFFNTT
   Consensus igqndgagaddlp!qqqgANdYaflsn..fNg##Dakr.VraainDaanrnt

            151       160       170       180       190       200
            |-----------+---------+---------+---------+---------|
   satA     LNGAKQILLFNLPDLGQNPSARSQKVYEAVSNVSAYHNKL-LLNLARQLA
   R.sol    AAATSGSGVTPAIATAQVQQAATDLVGYVKDNIAKGATQVYVFNLPDSSL
   Consensus aaaakqiglfnaialaQnqqAas#lVgeakdh!aaganql.llNLarqla

            201       210       220       230       240       250
            |-----------+---------+---------+---------+---------|
   satA     PTGNVKLFEIDKQFAENLRDPQNFGLSDVENPCYDGGYVNKPFATRSVST
   R.sol    TPDGVASGTTGQALLHALVGTFNTTLQSGLAGTSARIIDFNAQLTAAIQN
   Consensus ppdgValgeidqalaeaLrdpqNfgLqdgeagcsargidfnaqaTaa!qn

            251       260       270       280       290       300
            |-----------+---------+---------+---------+---------|
   satA     DRQLSAFSPQERLAIAG---NPLLAQAVASPN----ARRSASPLNCEGKNFN
   R.sol    GASFGFANTSARACDATKINALVPSAGGSSLFCSANTLVASGADQSYLFA
   Consensus daqlgaanpqaRaadAg..NaLlaqAgaSp$...Arrlaapgad#gk$Fa

            301       310       320       330
            |-----------+---------+---------|
   satA     DQVHPTTVVHAALSERAATFIETQYEFLAN
   R.sol    DGVHPTTAGHRLIASNVLARLLA---DNVAN
   Consensus DqVHPTTagHaaiaeraaariea..#nlAN
```

Figure 33

```
Pfam          *->ivafGDSltdggg...............ayygdsdgggwgagladrltsla..rlrargrgvdv
Sriml   38    .YVALGDSYSSGVG............agSYDSSSGSCKRSTKSYPALWAAS..------HTGTRF 81
Scoe1    5    YVAVGDSFTEG--..............--VGDPGPDGAFVGWADRLAVLL..ADRRPEGDFTY 47
Scoe2   10    LVAVGDSFTEG--..............--MSDLLPDGSYRGWADLLATRM..---AARSPGFRY 50
Scoe3  239    VVAFGDSITDG--..............ARSQSDAHHRWTDVLAARLHEAA..GDGRDTPRYSV 283
Scoe4   75    LHMLGDSTAAG--..............------QGVHRAGQTPGALLASG..LAAVAERPVRL 113
Scoe5   66.   VAAVGDSITRGFD............acAVLSDCPEVSWATGSSAKVDSLAvrLLGKADAAEHS 116
Ahyd1   28    IVMFGDSLSDTGKmyskmrgylpssppyYEGRPSNGPVWLEQLTNEFPGLTiaNEAEGGPTAVA 91
Asal1   28    IVMFGDSLSDTGKmyskmrgylpssppyYEGRPSNGPVWLEQLTKQF----..------PGLTI 79
Ahyd2   40    IVMFGDSLSDTGKmyskmrgylpssppyYEGRPSNGPVWLEQLTKQFPGLTiaNEAEGGATAVA 103


Pfam          fnrgisGrtsdGrlvvDarlvatllFlaqflGlnlpPYLsgdflrGANFAsagAtIlgtslipflni
Sriml   82    NFTACSGAR----------------------------------------------------------- 90
Scoe1   48    TNLAVRGRL----------------------------------------------------------- 56
Scoe2   51    ANLAVRGKL----------------------------------------------------------- 59
Scoe3  284    VNEGISGNR----------------------------------------------------------- 292
Scoe4  114    GSVAQPGAC----------------------------------------------------------- 122
Scoe5  117    WNYAVTGAR----------------------------------------------------------- 125
Ahyd1   92    YNKISWNPK------------------------------------------------------------ 100
Asal1   80    ANEAEGGAT----------------------------------------------------------- 88
Ahyd2  104    YNKISWNPK----------------------------------------------------------- 112


Pfam          QvqFkdfkskvlelrqa......lgllqellrlvpvldakspdlvtimiGtNDl...itvakfgpks
Sriml   91    ------------------......----TGDVLAKQLTPVNSGTDLVSITIGGNDAgfaDTMTTCNLQG 131
Scoe1   57    ------------------......--LDQIVAEQVPRVVGLAPDLVSFAAGGNDI...------I---- 86
Scoe2   60    ------------------......--IGQIVDEQVDVAAAMGADVITLVGGLNDT...--------- 88
Scoe3  293    --------LLTSRPGRPA......DNPSGLSRFQRDVLERTNVKAVVVVLGVNDV...---------- 333
Scoe4  123    ------------------......SDDLDRQVALVLAEPDRVPDICVIMVGANDV...---------- 153
Scoe5  126    ------------------......---MADLTAQVTRAAQREPELVAVMAGANDA...-------CR 155
Ahyd1  101    ------------YQVI......NNLDYEVTQFLQKDSFKPDDLVILWVGANDY...--------LA 137
Asal1   89    -------AVAYNKISWNpkyqvyNNLDYEVTQFLQKDSFKPDDLVILWVGANDY...--------LA 137
Ahyd2  113    ------------YQVI......NNLDYEVTQFLQKDSFKPDDLVILWVGANDY...--------LA 149


Pfam          .......tksdrnvsvpefrdnlrklikrLrsangariiilitlvllnlpl..........plGCl
Sriml  132    esaclarIAKARAYIQQTLPAQLDQVYDAIDSRAPAA-----QVVVLGYP-..........----- 176
Scoe1   87    .......---RPGTDPDEVAERFELAVAALT-AAAGTVLVTTGFDTRGVP-..........---- 125
Scoe2   89    .......---------LRPKCDMARVRDLLTQAVERLAPHCEQLVLMRSP-..........---- 122
Scoe3  334    .......LNSPELADRDAILTGLRTLVDRAHARGLRVVGATITPFGGYGG-..........----- 376
Scoe4  154    .......---THRMPATRSVRHLSSAVRRLR-TAGAEVVVGTCPDLGTIE-..........---- 192
Scoe5  156    .......STTSAMTPVADFRAQFEEAMATLR-KKLPKAQVYVSSIPDLKRLwsqgrtnplgkQVWKL 214
Ahyd1  138    .......YGWNTEQDAKRVRDAISDAANRMV-LNGAK-----EILLFNLP-..........----- 174
Asal1  138    .......YGWNTEQDAKRVRDAISDAANRMV-LNGAK-----QILLFNLP-..........---- 174
Ahyd2  150    .......YGWNTEQDAKRVRDAISDAANRMV-LNGAK-----QILLFNLP-..........----- 186


Pfam          pq.klalalassknvdatgclerlneavadynealrelaei.ek.l.q.aqlrkdglpdlkeanvpy
Sriml  177    --.RFYKLGGSCAVGLSEKSRAAINAAADDINAVTAKRA--.--.-.-.-----------ADBGFAF 219
Scoe1  126    --.----------VLKHLRGKIATYNGHVRAIA--.--.-.-.-----------DRYGCPV 152
Scoe2  123    --.----------GRQGPVLERFRPRMEALFAVIDDLA--.--.-.-.-----------GRHGAVV 154
Scoe3  377    --.YTEARETMRQEVNEEIRSGRVFDTVVDFDRALRDPY--.--.-.-.------------------- 412
Scoe4  193    --.-------------------------RVRQPLRWLaRRaSrQlAAAQTIGAVEQGGRTVSL 227
Scoe5  215    GLCPSMLGDADSLDSAATLRRNTVRDRVADYNEVLREVC-.--.-.-.AkDRRCRSDDGAVHEFRFGT 273
Ahyd1  175    --.----DLGQNPSARSQKVVEAASHVSAYHNQLLLNLA--.--.-.-.RQLAPTGMVKLFEIDKQF 224
Asal1  175    --.----DLGQNPSARSQKVVEAVSHVSAYHNKLLLALA--.--.-.-.RQLAPTGMVKLFSIDKQF 224
Ahyd2  187    --.----DLGQNPSARSQKVVEAVSHVSAYHNQLLLNLA--.--.-.-.RQLAPTGMVKLFSIDKQF 236


Pfam          VDlysifqdldgiqnpsayv.y....GPeet.kaCCGyGgr.yNyn.rv.CGnag.l.ck.vtakaC
Sriml  220    GDVNT---------------.-....-----.----------.-----.TFAgHRlCSGAPwL.HS.VT---- 242
Scoe1  153    LDLWSLRSVQDRRA------.-....-----.----------.-----.---.--.------.-.--.------ 166
Scoe2  155    VDLYGAQSLADPRM------.-....-----.----------.-----.---.--.------.-.--.------ 168
Scoe3  413    --------------------.-....-----.----------.-----.---.--.------.-.--.------ 413
Scoe4  228    GDLLGPEFAQNPREL------.-....-----.----------.-----.---.--.------.-.--.------ 242
```

```
Scoe5   274 DQL----------------.-.....----.------------.-----.--.------.-.--.------ 276
Ahyd1   225 AEMLRDPQNFGLSDQRNACYgGsyvwKPFASrSASTDSQLSaFNPQeRLaIAGNPllaQAvASPMAA 291
Asal1   225 AEMLRDPQNFGLSDVENPCYdGgyvwKPFATrSVSTDRQLSaFSPQeRLaIAGNPllaQAvASPMAR 291
Ahyd2   237 AEMLRDPQNFGLSDVENPCYdGgyvwKPFATrSVSTDRQLSaFSPQeRLaIAGNPllaQAvASPMAR 303


                                        ▼
Pfam        .dassyll.atlfwDgf.HpsekGykavAeal<-*
Srim1   243 .-------.--LPVENSyRPTAMGQSKGYLPV    263
Scoe1   167 .-------.--WDADRL.HLSPEGHTRVALRA    186
Scoe2   169 .-------.--WDVDRL.HLTAEGHRRVAEAV    188
Scoe3   413 .-DPRRMRaDYDSGDHL.RPGDKGYARMGAVI    441
Scoe4   243 .-------.--FGPDNY.RPSADGYATAAMAV    262
Scoe5   277 .-------.--SHWDWF.RPSVDGQARLAEIA    296
Ahyd1   292 rSASYLNCeGKMFWDQV.HPTTVVHAALSEPA    322
Asal1   292 rSASPLNCeGKMFWDQV.HPTTVVHAALSERA    322
Ahyd2   304 rSASPLNCeGKMFWDQV.HPTTVVHAALSERA    334
```

Figure 34

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                                    ▼
Pfam        *->ivafGDSltdggg...............ayygdsdgggwgagladrltsla..rlrargrgvdv
Sriml    38 YVALGDSYSSGVG.............agSYDSSSGSCKRSTKSYPALWAAS..------HTGTRF 81
Scoe1     5 YVAVGDSFTEG--...............--VGDPGPDGAFVGWADRLAVLL..ADERPEGDFTY 47
Scoe2    10 LVAVGDSFTEG--...............--MSDLLPDGSYRGWADLLATRM..--AARSPGFRY 50
Ahyd1    28 IVMFGDSLSDTGKmyskmrgylpssppyYEGRFSNGPVWLEQLTNEFPGLTiaNEAEGGPTAVA 91
Asal1    28 IVMFGDSLSDTGKmyskmrgylpssppyYEGRFSNGPVWLEQLTKQF----..------PGLTI 79
Ahyd2    40 IVMFGDSLSDTGKmyskmrgylpssppyYEGRFSNGPVWLEQLTKQFPGLTiaNEAEGGATAVA 103


Pfam        fnrgisGrtsdGrlvvDarlvatllFlaqflGlnlpPYLsgdflrGANPAsagAtIlgtslipflni
Sriml    82 NFTACSGAR---------------------------------------------------------- 90
Scoe1    48 TNLAVRGRL---------------------------------------------------------- 56
Scoe2    51 ANLAVRGKL---------------------------------------------------------- 59
Ahyd1    92 YNKISWNPK---------------------------------------------------------- 100
Asal1    80 ANEAEGGAT---------------------------------------------------------- 88
Ahyd2   104 YNKISWNPK---------------------------------------------------------- 112


                                                    ▼
Pfam        QvqFkdfkskvlelrqa......lgllqellrlvpvldakspdlvtimiGtNDl...itvakfgpks
Sriml    91 ----------------......---TGDVLAKQLTPVNSGTDLVSITIGGNDAgfaDTMTTCNLQG 131
Scoe1    57 ----------------......---LDQIVAEQVPRVVGLAPDLVSFAAGGNDI...------I---- 86
Scoe2    60 -----------------......---IGQIVDEQVDVAAAMGADVITLVGGLNDT..------------ 88
Ahyd1   101 ------------YQVI......NNLDYEVTQFLQKDSFKPDDLVILWVGANDY...-------LA 137
Asal1    89 -------AVAYNKISWNpkyqvyNNLDYEVTQFLQKDSFKPDDLVILWVGANDY...-------LA 137
Ahyd2   113 ------------YQVI......NNLDYEVTQFLQKDSFKPDDLVILWVGANDY...-------LA 149


Pfam        .......tksdrnvsvpefrdnlrklikrLrsangariiilitlvllnlplplGCl
Sriml   132 esaclarIAKARAYIQQTLPAQLDQVYDAIDSRAPAA------QVVVLGYP------ 176
Scoe1    87 .......---RPGTDPDEVAERFELAVAALT-AAAGTVLVTTGFDTRGVP------ 125
Scoe2    89 .......----------LRPKCDMARVRDLLTQAVERLAPHCEQLVLMRSP------ 122
Ahyd1   138 .......YGWNTEQDAKRVRDAISDAANRMV-LNGAK------RILLFNLP------ 174
Asal1   138 .......YGWNTEQDAKRVRDAISDAANRMV-LNGAK------QILLFNLP------ 174
Ahyd2   150 .......YGWNTEQDAKRVRDAISDAANRMV-LNGAK------QILLFNLP------ 186


Pfam        pqklalalalassknvdatgclerlneavadynealrelaeieklqaqlrkdglpdlkeanvpy
Sriml   177 --RFYKLGGSCAVGLSEKSRAAINAAADDINAVTAKRA-----------------ADHGFAF 219
Scoe1   126 ---------------VLKHLRGKIATYNGHVRAIA----------------DRYGCPV 152
Scoe2   123 ---------------GRQGPVLERFRPRMEALFAVIDDLA----------------GRHGAVV 154
Ahyd1   175 ------DLGQNPSARSQKVVEAASHVSAYHNQLLLNLA------RQLAPTGMVKLFEIDKQF 224
Asal1   175 ------DLGQNPSARSQKVVEAVSHVSAYHNKLLLNLA------RQLAPTGMVKLFEIDKQF 224
Ahyd2   187 ------DLGQNPSARSQKVVEAVSHVSAYHNQLLLNLA------RQLAPTGMVKLFEIDKQF 236


                                  ▼
Pfam        VDlysifqdldgignpsayv.y....GFeet.kaCCGyGgr.yNyn.rv.CGnag.l.ck.vtakaC
Sriml   220 GDVRT-------------.-....----.---------.-TFAgHElCSGAPwL.HS.VT---- 242
Scoe1   153 LDLWSLRSVQDRRA------.-....----.---------.----.--.-----.-.--.------ 166
Scoe2   155 VDLYGAQSLADPRM-------.-....----.---------.----.--.-----.-.--.------ 168
Ahyd1   225 AEMLRDPQNFGLSDQRNACYgGsyvwKPFASrSASTDSQLSaFNPQeRLaIAGNPlLaQAvASPMAA 291
Asal1   225 AEMLRDPQNFGLSDVENPCYdGgyvwKPFATrSVSTDRQLSaFSPQeRLaIAGNPlLaQAvASPMAR 291
Ahyd2   237 AEMLRDPQNFGLSDVENPCYdGgyvwKPFATrSVSTDRQLSaFSPQeRLaIAGNPlLaQAvASPMAR 303


                    ▼
Pfam        .dassyll.atlfwDgf.HpsekGykavAeal<-*
Sriml   243 .------.--LPVENSyHPTANGQSKGYLPV     263
Scoe1   167 .------.--WDADRL.HLSPEGHTRVALRA     186
Scoe2   169 .------.--WDVDRL.HLTAEGHRRVAEAV     188
Ahyd1   292 rSASTLNCeGKMFWDQV.HPTTVVHAALSEPA   322
Asal1   292 rSASPLNCeGKMFWDQV.HPTTVVHAALSERA   322
Ahyd2   304 rSASPLNCeGKMFWDQV.HPTTVVHAALSERA   334
```

Figure 35

pET12-AsalGCAT=pSM
5619 bp
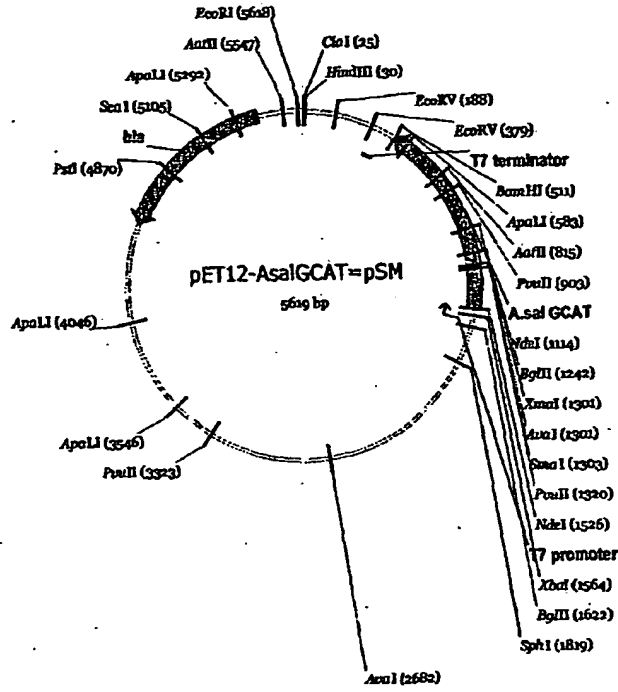
Figure 36

Figure 37

Figure 38

Degradation products

Samples spotted

| 1 | 2 | 3 | 4 | 5 |

Figure 39

Controls

Pos   Neg   SE   His   SE   His   SE   His   SE   His

A. hydrophila enzyme   A. salmonicida

enzyme

Figure 40.

pet12a-A.h. GCAT=pSMa
5640 bp

Figure 41:

Figure 43

Controls
Pos   neg   A. hydrophila GCAT   A. sal GCAT

SE   His   SE   His   SE   His   SE   His

Figure 44

38

Figure 45

Lyso-phosphatidylcholine

Phosphatidylcholine

Phosphatidylethanolamine

9   8   7   6   5   4   3   2   1

Figure 46

Cholesterol-and sitosterolester
Triglyceride

Free fatty acid

Cholesterol + sitosterol

1 2 3 4 5 6 7 8 9

Figure 47

Phosphatidylcholine

Cholesterol

Transferase

Cholesterolester

Lyso-phosphatidylcholine

Figure 48

TLC solvent I

Cholesterol esters
Triglyceride

Fatty acids

Monoglyceride

6 7 8 9   6 7 8 9   6 7 8 9

1 h   2 h   4 h

Figure 49

Figure 50

Cholesterolester

Fatty acid

Cholesterol

Phosphatidylcholine

C1	2	3	4	5	6	C7	Chl	Chl	Chl
Min. 30	30	60	150	240	156	1560		ester	

Figure 51

2254-82-5. TLC of egg yolk lipid treated with transferase #135

| Min.30 | 30 | 60 | 150 | 240 | 1560 | 1560 | | ester |

Cholesterolester
Cholesterol
Fatty acid

Phosphatidylethanolamine

Phosphatidylcholine

lyso-Phosphatidylcholine

Figure 52

Lipid from enzyme treated egg yolk TLC solv. IV

Figure 53

The amount of fatty acid or cholesterol ester produced as a function of time
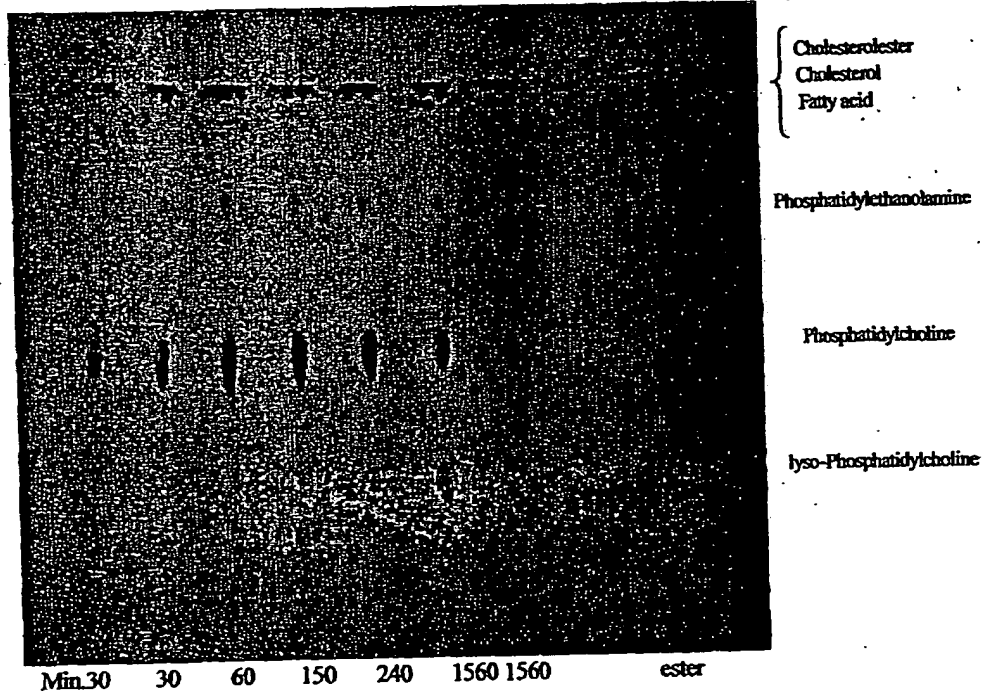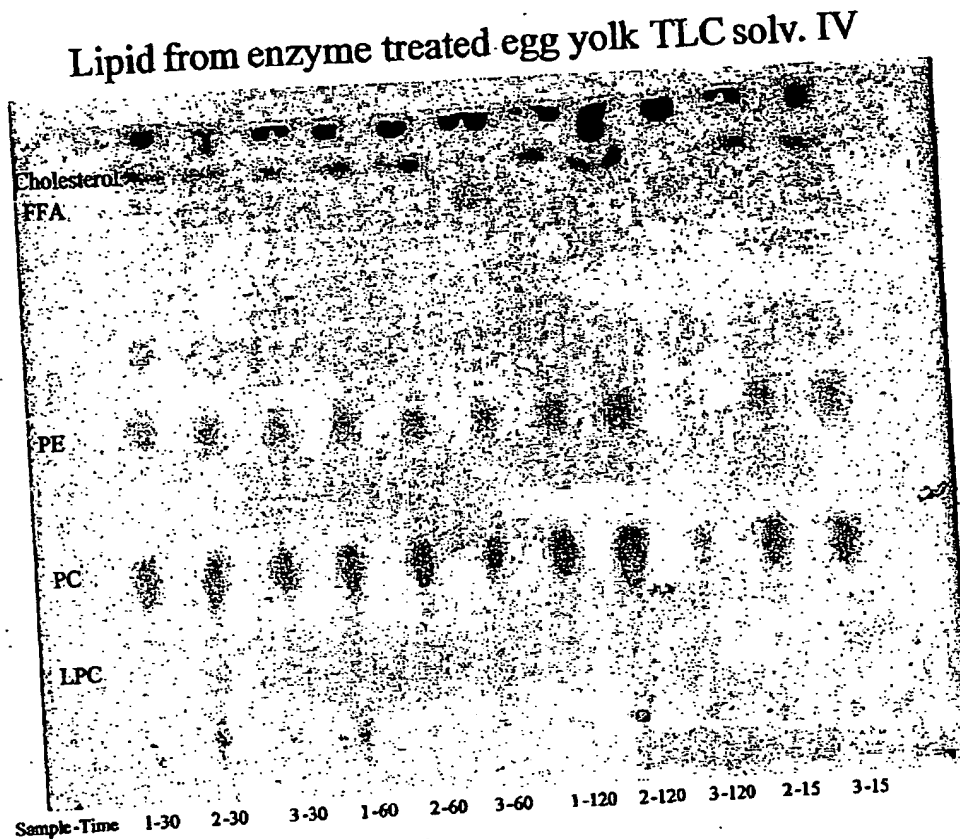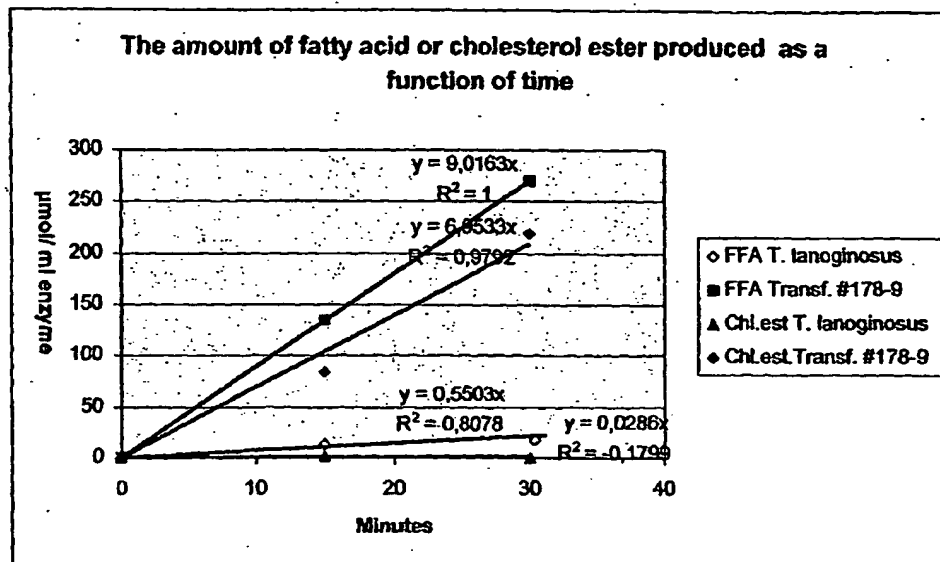
Figure 54

Relative transferase activity as % of transferase and hydrolytic
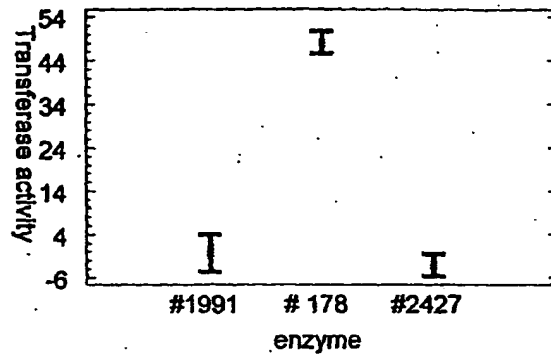
Figure 55

Transferase activity for #178 as a function of % water in the assay.
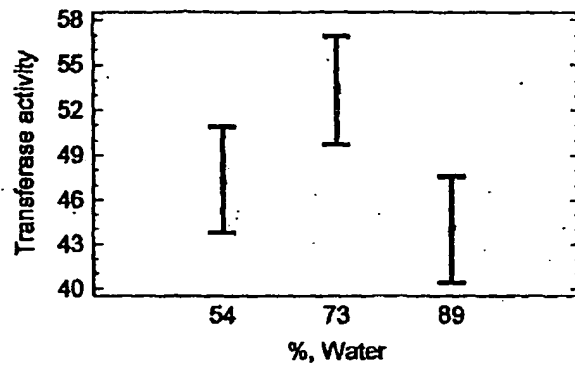
Figure 56 .

Transferase activity for #178 as a function of reaction time the assay.

Figure 57

GLC analysis of fatty acid and cholesterol

Figure 58

GLC analysis of fatty acid and

$y = 2,5178x + 37,272$
$R^2 = 0,974$

$y = 0,8372x + 77,383$
$R^2 = 0,9935$

◆ Fatty acid

■ Cholesterol ester

—— Linear (Fatty acid)

- - - Linear (Cholesterol ester)

y-axis: µmol/ml enzyme (40,0 — 200,0)
x-axis: Minutes (10 — 60)
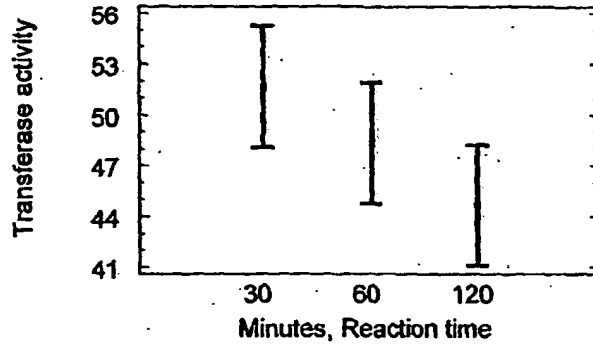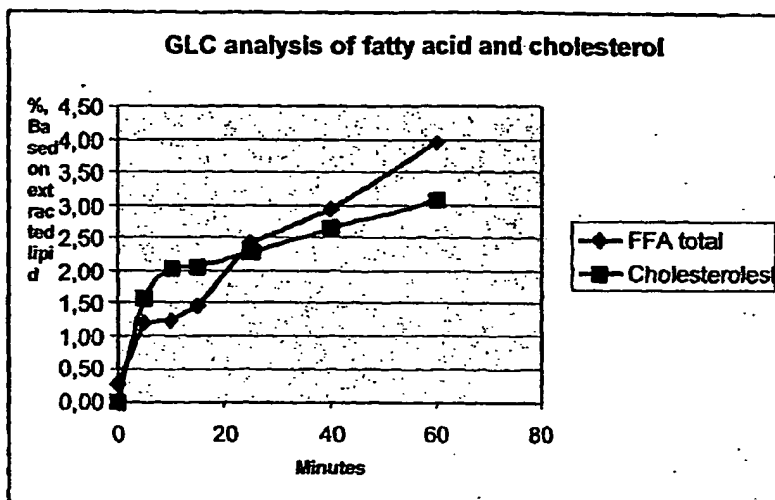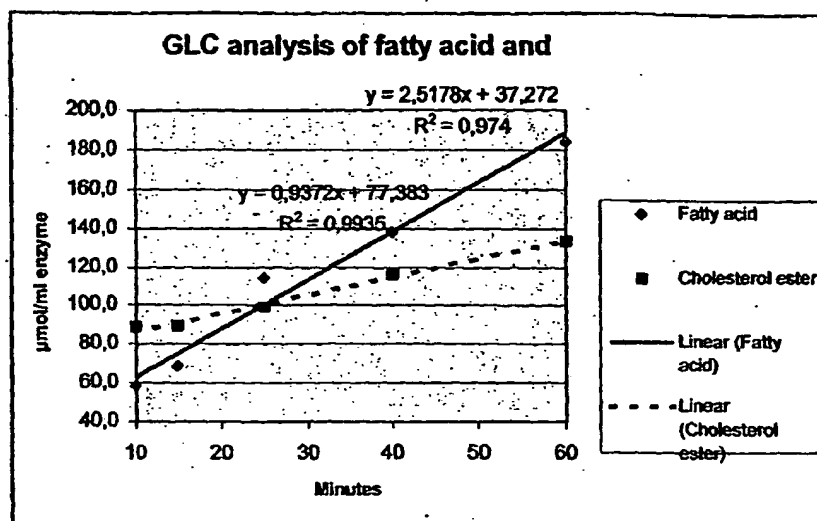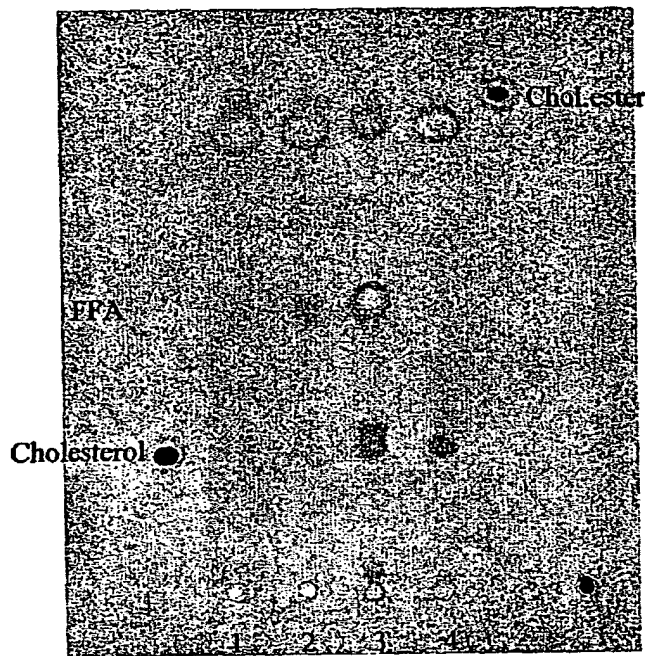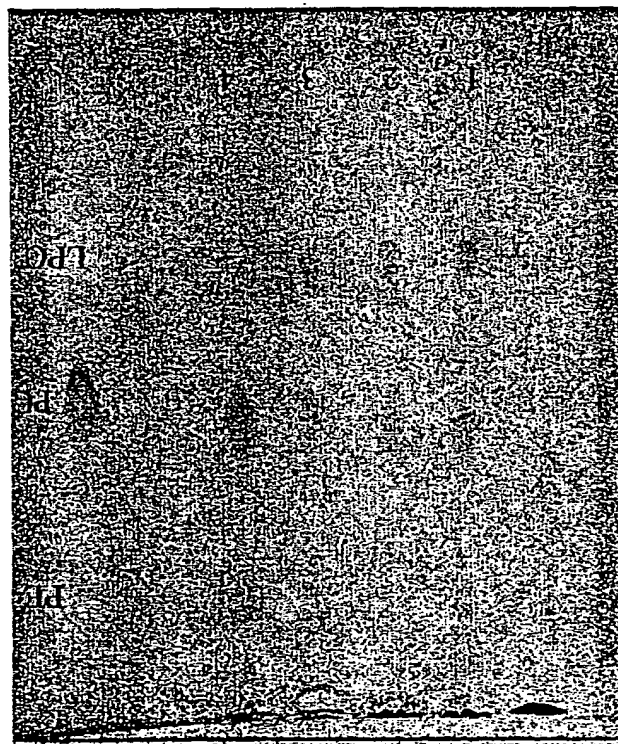
Figure 59

Figure 60.

Figure 61
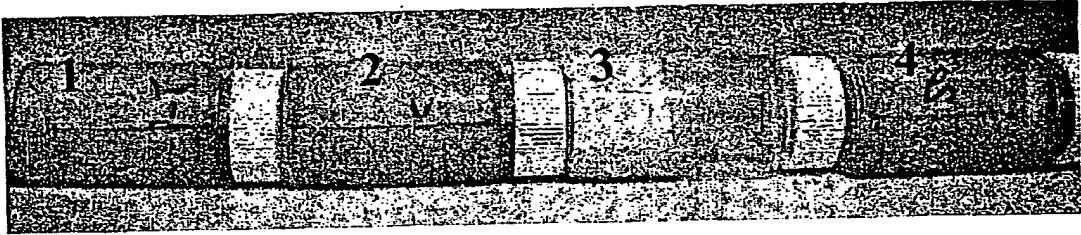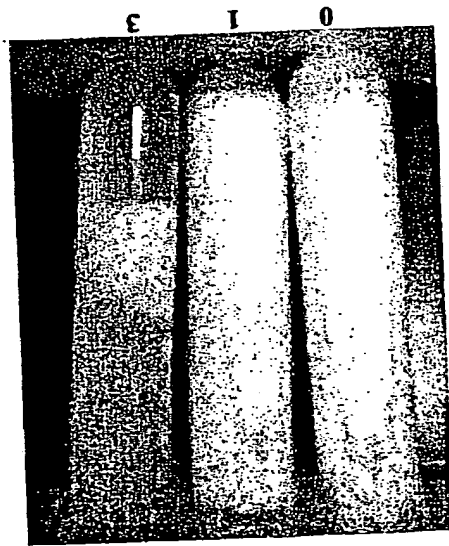
Figure 62
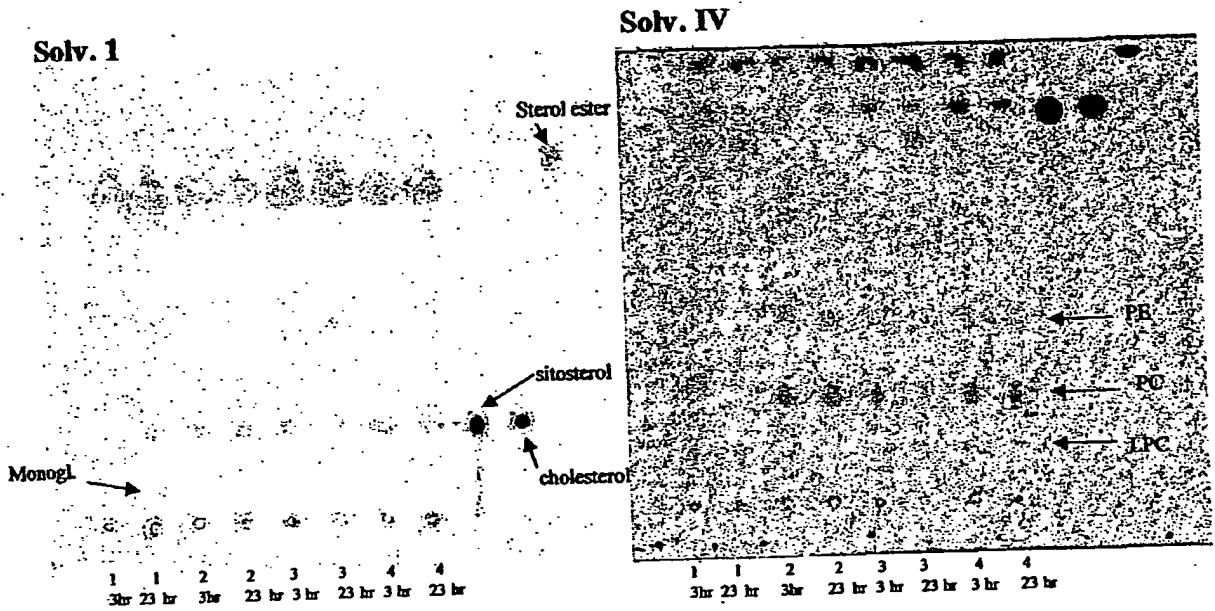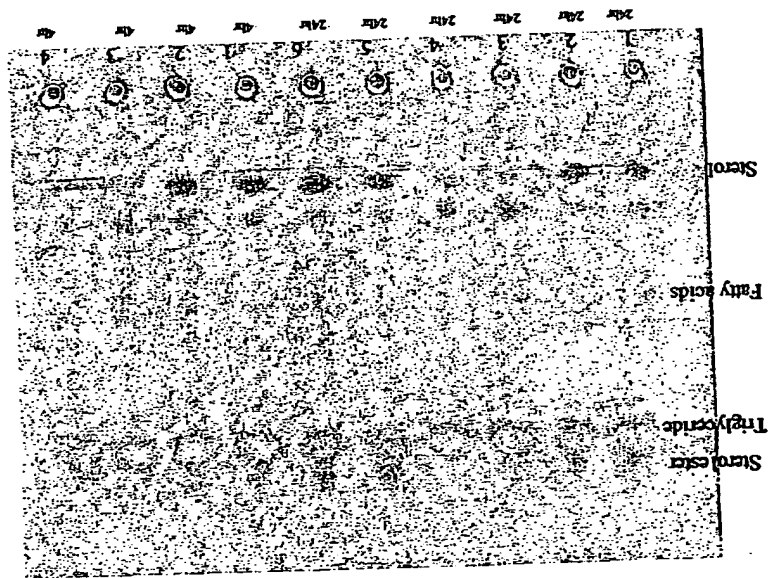
Figure 63

Figure 64

Figure 65
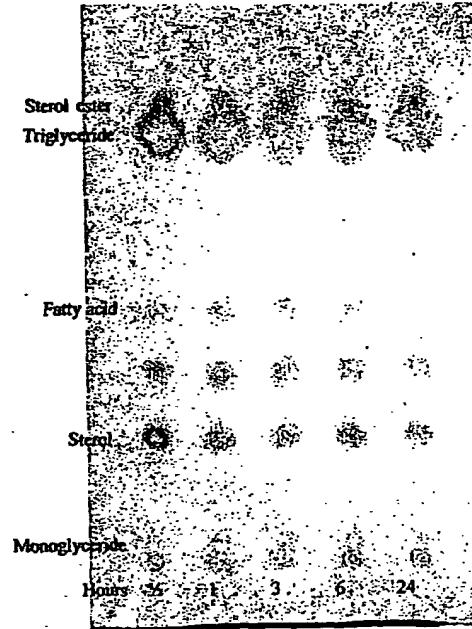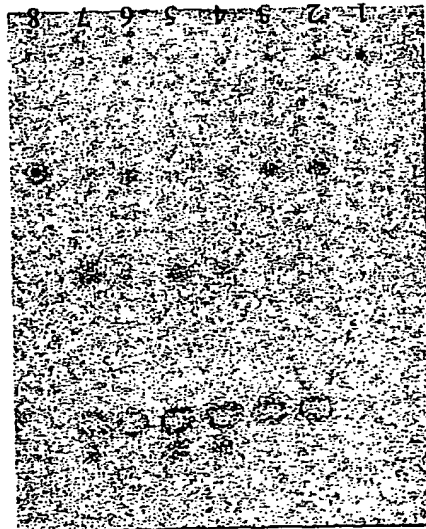
Figure 66

Figure 67

Figure 68

Figure 69

PE

PC

LPC

1 2 3 4 5 6 7 8 9 10 11

Figure 70

DGDG   sucrose

DGMG   Sucrose ester
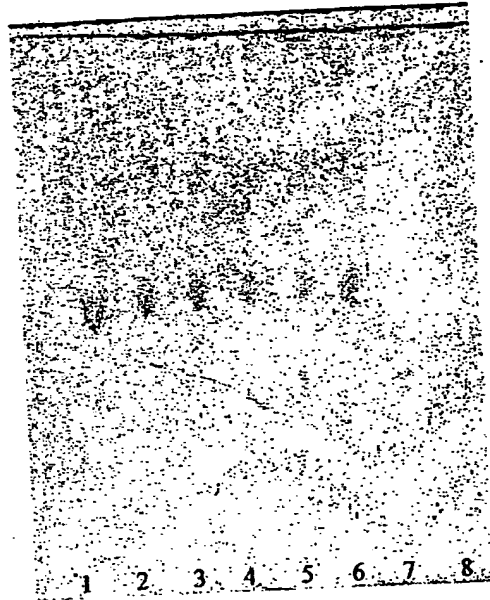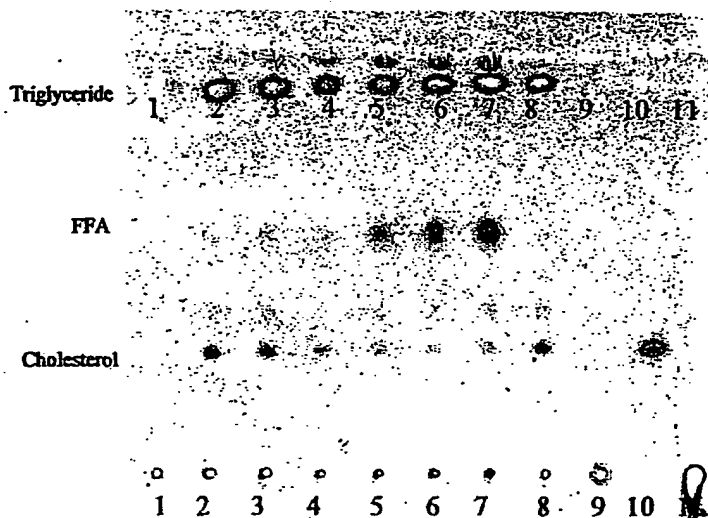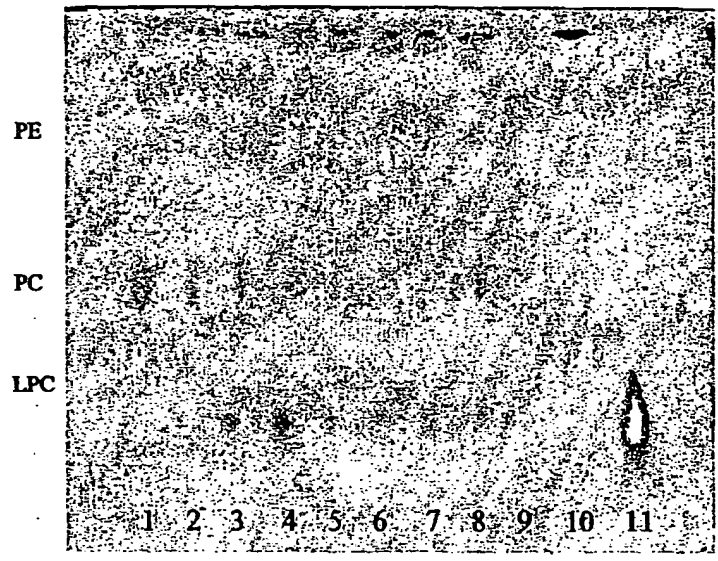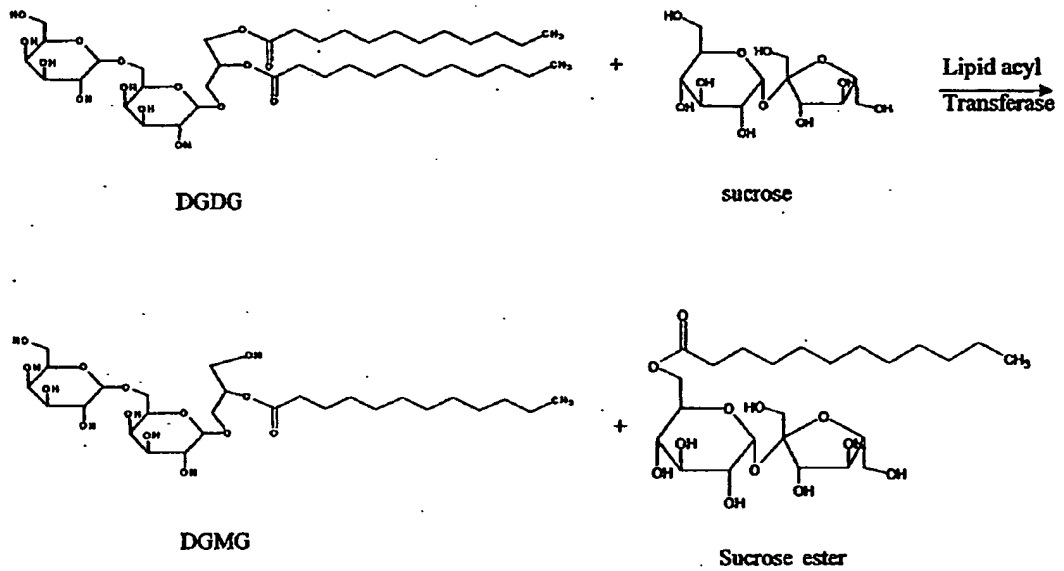
Figure 71

(SEQ ID No. 36)

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  1  MFKFKKNFLV GLSAALMSIS LFSATASAAS ADSRPAFSRI VMFGDSLSDT
 51  GKMYSKMRGY LPSSPPYYEG RFSNGPVWLE QLTKQFPGLT IANEAEGGAT
101  AVAYNKISWN PKYQVINNLD YEVTQFLQKD SFKPDDLVIL WVGANDYLAY
151  GWNTEQDAKR VRDAISDAAN RMVLNGAKQI LLFNLPDLGQ NPSARSQKVV
201  EAVSHVSAYH NQLLLNLARQ LAPTGMVKLF EIDKQFAEML RDPQNFGLSD
251  VENPCYDGGY VWKPFATRSV STDRQLSAFS PQERLAIAGN PLLAQAVASP
301  MARRSASPLN CEGKMFWDQV HPTFVVHAAL SERAATFIAN QYEFLAH**
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Figure 72 (SEQ ID No. 54)

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   1  ATGTTTAAGT TTAAAAAGAA TTTCTTAGTT GGATTATCGG CAGCTTTAAT
      TACAAATTCA AATTTTTCTT AAAGAATCAA CCTAATAGCC GTCGAAATTA

  51  GAGTATTAGC TTGTTTTCGG CAACCGCCTC TGCAGCTAGC GCCGACAGCC
      CTCATAATCG AACAAAAGCC GTTGGCGGAG ACGTCGATCG CGGCTGTCGG

 101  GTCCCGCCTT TTCCCGGATC GTGATGTTCG GCGACAGCCT CTCCGATACC
      CAGGGCGGAA AAGGGCCTAG CACTACAAGC CGCTGTCGGA GAGGCTATGG

 151  GGCAAAATGT ACAGCAAGAT GCGCGGTTAC CTCCCCTCCA GCCCGCCCTA
      CCGTTTTACA TGTCGTTCTA CGCGCCAATG GAGGGGAGGT CGGGCGGGAT

 201  CTATGAGGGC CGTTTCTCCA ACGGACCCGT CTGGCTGGAG CAGCTGACCA
      GATACTCCCG GCAAAGAGGT TGCCTGGGCA GACCGACCTC GTCGACTGGT

 251  AACAGTTCCC GGGTCTGACC ATCGCCAACG AAGCGGAAGG CGGTGCCACT
      TTGTCAAGGG CCCAGACTGG TAGCGGTTGC TTCGCCTTCC GCCACGGTGA

 301  GCCGTGGCTT ACAACAAGAT CTCCTGGAAT CCCAAGTATC AGGTCATCAA
      CGGCACCGAA TGTTGTTCTA GAGGACCTTA GGGTTCATAG TCCAGTAGTT

 351  CAACCTGGAC TACGAGGTCA CCCAGTTCTT GCAGAAAGAC AGCTTCAAGC
      GTTGGACCTG ATGCTCCAGT GGGTCAAGAA CGTCTTTCTG TCGAAGTTCG

 401  CGGACGATCT GGTGATCCTC TGGGTCGGTG CCAATGACTA TCTGGCCTAT
      GCCTGCTAGA CCACTAGGAG ACCCAGCCAC GGTTACTGAT AGACCGGATA

 451  GGCTGGAACA CGGAGCAGGA TGCCAAGCGG GTTCGCGATG CCATCAGCGA
      CCGACCTTGT GCCTCGTCCT ACGGTTCGCC CAAGCGCTAC GGTAGTCGCT

 501  TGCGGCCAAC CGCATGGTAC TGAACGGTGC CAAGCAGATA CTGCTGTTCA
      ACGCCGGTTG GCGTACCATG ACTTGCCACG GTTCGTCTAT GACGACAAGT

 551  ACCTGCCGGA TCTGGGCCAG AACCCGTCAG CTCGCAGTCA GAAGGTGGTC
      TGGACGGCCT AGACCCGGTC TTGGGCAGTC GAGCGTCAGT CTTCCACCAG

 601  GAGGCGGTCA GCCATGTCTC CGCCTATCAC AACCAGCTGC TGCTGAACCT
      CTCCGCCAGT CGGTACAGAG GCGGATAGTG TTGGTCGACG ACGACTTGGA

 651  GGCACGCCAG CTGGCCCCCA CCGGCATGGT AAAGCTGTTC GAGATCGACA
      CCGTGCGGTC GACCGGGGGT GGCCGTACCA TTTCGACAAG CTCTAGCTGT

 701  AGCAATTTGC CGAGATGCTG CGTGATCCGC AGAACTTCGG CCTGAGCGAC
      TCGTTAAACG GCTCTACGAC GCACTAGGCG TCTTGAAGCC GGACTCGCTG

 751  GTCGAGAACC CCTGCTACGA CGGCGGCTAT GTGTGGAAGC CGTTTGCCAC
      CAGCTCTTGG GGACGATGCT GCCGCCGATA CACACCTTCG GCAAACGGTG

 801  CCGCAGCGTC AGCACCGACC GCCAGCTCTC CGCCTTCAGT CCGCAGGAAC
      GGCGTCGCAG TCGTGGCTGG CGGTCGAGAG GCGGAAGTCA GGCGTCCTTG

 851  GCCTCGCCAT CGCCGGCAAC CCGCTGCTGG CACAGGCCGT TGCCAGTCCT
      CGGAGCGGTA GCGGCCGTTG GGCGACGACC GTGTCCGGCA ACGGTCAGGA

 901  ATGGCCCGCC GCAGCGCCAG CCCCCTCAAC TGTGAGGGCA AGATGTTCTG
      TACCGGGCGG CGTCGCGGTC GGGGGAGTTG ACACTCCCGT TCTACAAGAC

 951  GGATCAGGTA CACCCGACCA CTGTCGTGCA CGCAGCCCTG AGCGAGCGCG
      CCTAGTCCAT GTGGGCTGGT GACAGCACGT GCGTCGGGAC TCGCTCGCGC

1001  CCGCCACCTT CATCGCGAAC CAGTACGAGT TCCTCGCCCA CTGATGA
      GGCGGTGGAA GTAGCGCTTG GTCATGCTCA AGGAGCGGGT GACTACT
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Figure 73